KU LEUVEN

ARENBERG DOCTORAL SCHOOL
FACULTY OF BIOSCIENCE ENGINEERING

Design of a whole-cell bioreporter for the construction of a hormone biosensor

Seppe Dierckx

Supervisors:
Prof. J. Vanderleyden
Dr. W. Eberle
Prof. G. Gielen

Dissertation presented in partial fulfillment of the requirements for the degree of Doctor in Bioscience Engineering

December 2015

Design of a whole-cell bioreporter for the construction of a hormone biosensor

Seppe DIERCKX

Supervisory Committee:
Prof. J. Buyse (KU Leuven), chair
Prof. J. Vanderleyden, supervisor (KU Leuven)
Dr. W. Eberle, co-supervisor (Imec)
Prof. G. Gielen, co-supervisor (KU Leuven)
Prof. J. Michiels (KU Leuven)
Prof. D. Springael (KU Leuven)
Prof. J. Robben (KU Leuven)
Dr. W. Van Roy (Imec)

Dissertation presented in partial fulfillment of the requirements for the degree of Doctor in Bioscience Engineering

December 2015

Doctoraatsproefschrift nr. 1318 aan de faculteit Bio-ingenieurswetenschappen van de KU Leuven

Uitgegeven in eigen beheer, Seppe Dierckx, Kasteelpark Arenberg 20, B-3001 Heverlee (Belgium)

Dankwoord

I have not failed. I've just found
10,000 ways that won't work.

Thomas A. Edison

Het bovenstaande citaat is een beetje het motto geworden van mijn doctoraat aangezien ik doorheen de jaren minstens evenveel vruchtbare als vruchtloze ideeën heb gehad. Voor elk succesvol experiment waren er ook een aantal geflopte, en voor elk gelukt construct waren er daarvoor al tientallen mislukt. Het doctoraat werd al gauw eerder een last op de motivatie dan op de fysieke of mentale bekwaamheid. Zo ontstond in de vriendengroep al snel het gezegde "Een doctoraat is vooral een beproeving voor je uithoudingsvermogen, niet je intelligentie". Het boekje dat je nu in handen hebt is het resultaat van mijn uithoudingsvermogen, bijgestaan door een hele hoop mensen die ik bij deze wil bedanken.

Eerst en vooral bedankt Jos om mij alle kansen te geven om te geraken waar ik nu ben. Bij aanvang had ik enkel theoretische kennis over synthetische biologie en alles wat met bacteriën te maken heeft. Die heb jij aangevuld met je vertrouwen en steun. En vooral die laatste twee hebben voor het welslagen van deze onderneming gezorgd.
In die lijn wil ik ook graag Wolfgang bedanken voor de ondersteuning bij de meer technische aspecten van dit werk.

Ik zou ook graag de leden van de examencommissie bedanken voor het aanreiken van de suggesties ter verbetering van dit doctoraatsmanuscript.

Daarnaast wil ik ook Imec bedanken voor de financiële ondersteuning om dit onderzoek te verrichten.

Anita, ik kan het aantal keren dat jij mij gered hebt van een of andere administratieve nachtmerrie al lang niet meer tellen op één hand. Daarvoor,

en voor het bezorgen van alle pakjes in onze bureau, zou ik je graag willen bedanken. Jos, bedankt voor alle technische ondersteuningen, het in stand houden van de orde en regelgeving in het labo en voor het actief meedenken aan de technische invulling van het project van mijn masterthesisstudent. Ook een gemeende dank u wel aan André voor alles wat ik over de jaren heb stuk gemaakt met een glimlach te repareren.

De collega's oefenen een sterke invloed uit op het uithoudingsvermogen. Gelukkig kon ik rekenen op de steun en hulp van zeer fijne collega's binnen S&P. Vooral Tine, David en Ami, bedankt om mijn theoretische kennis over alles wat met bacteriën te maken heeft om te zetten in praktische kennis.
Sandra, zonder jou was mijn paper waarschijnlijk nog altijd niet aanvaard en was ik nu ongetwijfeld nog de inleiding van mijn doctoraatsthesis aan het schrijven. Dank u om mij te motiveren in de laatste stappen van het doctoraat. De synthetische biologische basis is mij bijgebracht door Lyn, die ik daar dan ook voor wil bedanken. Alsook voor alle congresmomenten in exotische en minder exotische locaties. En uiteraard ook om mij te introduceren in de wereld van de bacteriële doolhoven waarbij we zeer veel hebben bijgeleerd over verdamping en sealing (en veel hebben kunnen roddelen).
Ook de rest van S&P wil ik graag bedanken voor de sfeer in het labo, leuke laboweekends en gezellige etentjes, bedankt dus Lise, Elke V, Elke L, Stefanie, Elien, Ilse, Kenny, Ansie, Marijke, Cynthia, Hanne, Ingmar, Hans, Irina en Kai. Nog een extra bedankt aan Stijn voor de laatste naleessessies en om mij te helpen het mannelijk tegengewicht te vormen tijdens de trip naar Cork. Aurélien, bedankt om steeds klaar te staan met uitgebreide plannen over het verloop van jouw masterthesis. Je onderwerp was allesbehalve traditioneel maar we hebben er samen iets moois van gemaakt, grotendeels door jouw enthousiasme.

Voorts nog een speciale vermelding voor mijn bureaugenoten. Ik heb over de jaren redelijk wat bureaugenoten versleten maar met de laatste was de band toch iets sterker. Waarschijnlijk omdat we toevallig met vier tegelijk ons doctoraat hebben afgewerkt, bedankt voor al de steun- en lachmomenten Mariya, Bram, Kim en Patrizia. Bedankt ook Kim voor het opsommen van alle administratie die ik anders ongetwijfeld vergeten zou zijn, bedankt Bram voor alle hulp met statistiek en om samen met mij Kim te ambeteren. Natuurlijk ben ik onze bureau-postdoc ook heel dankbaar, dank u Mariya om ons bij te staan met wijze raad en nodige verluchting in stressmomenten. Jouw afwezigheid in de bureau was uiterst merkbaar tijdens je verblijf in Canada, ik ben dan ook zeer blij dat je net op tijd terug was voor mijn laatste weken.

Ik heb over de jaren ook veel plezier beleefd op het CMPG. Feestjes, festivals, sportwedstrijden, loopwedstrijden, etentjes, recepties, spelletjesavonden, WK-voetbalwedstrijden, Wii-avonden, ... En daarom ook een dikke merci aan Sparkles, Wouter, Nicolas, Joran, Toon, Dorien, Shanjun, Liselot, Maarten

F, Maarten G, Evelien, Ann, Kristine, Veerle, Pieter, Valerie, Tanne, Katrijn, Taiyeb, Vincent, Natalie, Cyrielle, Els, Katy, Sara, ...
In het bijzonder ook bedankt aan de lopers, de dinsdagmiddag zal in mijn hoofd nog lang dé loopmiddag zijn. Ook de langere afstanden op zondagochtend zullen mij bijblijven. En uiteraard ook bedankt aan de boyz voor de pauzekes en de vriendschap.

Na de werkuren kon ik ook altijd op de steun rekenen van mijn medebiochemisten. De woensdagavondetentjes waarop we ons beklag konden doen en steun konden uitdelen, gaven steeds hernieuwde moed voor de volgende dagen (toch op zijn minst tot aan het weekend). Ook de weekendjes weg, ski- of zonvakanties waren overgetelijk. Dank u wel voor jullie vriendschap en steun. Ik vrees dat ik dan toch de wedstrijd heb verloren ...

Ook mijn Kempische vrienden wil ik graag bedanken voor de friet-en-café-avonden, zaalvoetbal en slaapgelegenheid bij blitzbezoek in het buitenland. Bedankt om er altijd te zijn. Alsook bedankt aan de friendies voor het regelmatig aanhoren van geklaag en al te fantastische verhalen.

Ik zou ook graag mijn ouders bedanken voor alle kansen die ze mij hebben gegeven in het leven. Evenals voor het begrip voor mijn keuze om in het Leuvense te blijven plakken. Finne en Jens, bedankt voor jullie geduld als ik probeerde uitleggen waar ik mee bezig was. Alsook voor de steun, de spelletjesavonden/nachten en het enthousiasme bij het helpen uitdenken en uitvoeren van mijn receptie.
Tevens zou ik ook graag Jan, Christa, Thomas, Danitza, Andries en Margot bedanken voor de constante steun, etentjes en het spelen van vele spelletjes.

Zoals steeds heb ik het beste voor het laatste bewaard, of eerder de beste. Astrid, dank je om vijf jaar lang al mijn gezeur te tolereren en al mijn moodswings te aanvaarden. Voor het begrijpen van mijn regelmatige mentale afwezigheid wanneer ik thuis nog een of ander raadsel probeerde op te lossen in mijn hoofd. Voor het luisteren naar mogelijkse oplossingen voor die raadsels, gevolgd door een aantal redenen waarom het slechte oplossingen zijn. Voor de ontspannende reisjes tussendoor waardoor ik er telkens weer helemaal tegen kon. Voor de poging om het beste verjaardagscadeau ooit te organiseren (stomme oktoberse regenbuien!). Voor de talloze gezellige avonden thuis.
Maar vooral bedankt omdat je er altijd bent voor mij.

Seppe

Abstract

Recent research has shown that the increased use of oral contraceptives leads to an accumulation of steroid hormones in surface water all over the world. Both animals living in surface waters and human beings are possibly affected by the presence of these steroid hormones. In order to gauge the severity of the problem, an adequate sensor for steroids hormones is necessary since as of now, no *in situ* sensors are available.

Bacteria, engineered to detect specific molecules, so-called bioreporters offer an elegant solution for the detection of steroid hormones. They are rapid, easy to use systems that produce a measurable signal in the presence of specific molecules, mostly environmental pollutants.
A bioreporter that is integrated with a transducer that can measure the signal the bioreporter is emitting, is called a biosensor and can be used as a self-contained analytical measuring system.

This work describes the development of four different whole-cell bacterial hormone bioreporters. Two auxin bioreporters were constructed as a proof of principle using the HpaA transcription factor as input module. HpaA, a transcriptional regulator from *Escherichia coli*, was combined with two different output modules resulting in a GFP-producing fluorescent bioreporter and a pyocyanin-producing bioreporter. The latter was integrated with a multielectrode array to result in an electrochemical biosensor.
Both the fluorescent auxin bioreporter and the electrochemical auxin biosensor are able to dose-responsely detect auxins 2-phenylacetic acid and 4-hydroxyphenylacetic acid concentrations as low as 31 μM for the fluorescent biosensor and 2 μM for the electrochemical biosensor. Indole-3-acetic acid cannot be detected by either biosensor.

To construct the two steroid hormone bioreporters, transcriptional regulator RepA from *Comamonas testosteroni* was combined with a GFP and a pyocyanin output module.

The GFP-producing steroid hormone bioreporter is able to dose-responsely detect testosterone concentrations as low as 250 μM, and 17β-estradiol , 17α-ethinylestradiol, estrone and progesterone concentrations as low as 1 mM. A functional pyocyanin-producing steroid hormone bioreporter could not be constructed.

The biosensors constructed in this work are able to dose-responsely detect auxins and steroid sex hormones. However, the limit of detection is relatively high and can still be improved in the future.

Samenvatting

Recent onderzoek heeft aangetoond dat het groeiend verbruik van orale contraceptiva leidt tot een opstapeling van steroïdhormonen in oppervlaktewater overal in de wereld. Zowel dieren die in oppervlaktewater leven als mensen die dit water dagelijks gebruiken, worden dan ook mogelijk beïnvloed door de aanwezige steroïdhormonen. Om de grootte van het probleem te kunnen inschatten is er nood aan een aangepaste methode om de concentratie van steroïdhormonen in oppervlaktewaters te bepalen. Momenteel gebeurt dit met behulp van massaspectrometrie en chromatografie maar een *in situ* sensor dringt zich op.

Bacteriën die zodanig gemodificeerd zijn dat ze specifieke moleculen kunnen detecteren, ook wel bioreporters genoemd, bieden een elegante oplossing voor het detecteren van steroïdhormonen. Het zijn snelle, eenvoudig te gebruiken systemen die een meetbaar signaal produceren in de aanwezigheid van specifieke moleculen, meestal milieuvervuilende chemicaliën.
Wanneer men een bioreporter integreert met een transductor, die het signaal dat de bioreporter produceert kan detecteren, spreekt men van een biosensor. Een biosensor kan gebruikt worden als een onafhankelijk analytisch meetsysteem.

Deze studie beschrijft de ontwikkeling van vier verschillende cellulaire bacteriële hormoonbioreporters. Vooreerst zijn er twee auxinebioreporters ontwikkeld als een proof of principle door de transcriptiefactor HpaA als inputmodule te gebruiken. HpaA, afkomstig van *Escherichia coli*, werd gecombineerd met twee verschillende output-modules. Dit resulteerde in een GFP-producerend fluorescente bioreporter en een pyocyanine-producerende bioreporter. De laatste is dan geïntegreerd met een microelectrode om een elektrochemische biosensor te bekomen.
Beide auxinebioreporters kunnen de auxines fenylacetaat en hydroxyfenylacetaat concentraties vanaf 31 μM voor de fluorescente bioreporter en vanaf 2 μM voor de elektrochemische biosensor meten. Geen van beide bioreporters kan indole-3-acetaat detecteren.

Voor de constructie van de twee steroïdhormoonbioreporters werd transcriptiefactor RepA van *Comamonas testosteroni* gecombineerd met een GFP-en een pyocyanine-outputmodule.
De GFP-producerende steroïdhormoonbioreporter kan testosteron detecteren vanaf een concentratie van 250 μM, en 17β-estradiol, 17α-ethinylestradiol, estron en progesteron vanaf 1mM. Een functionele pyocyanine-producerende steroïdhormoonbiosensor kon niet ontwikkeld worden.

The bioreporters ontwikkeld tijdens deze studie vertonen een dosis/respons relatie bij het detecteren van auxines en steroïdhormonen. Maar voor alle reporters ligt de detectielimiet redelijk hoog en deze moet in de toekomst nog verbeterd worden.

Abbreviations

C. testosteroni	*Comamonas testosteroni*
E. coli	*Escherichia coli*
P. aeruginosa	*Pseudomonas aeruginosa*
ARE	Androgen Response Element
BPA	Bisphenol A
BSA	Bovine Serum Albumin
4-Cl-IAA	4-Chloroindole-3-acetic Acid
COSS	*Comamonas testosteroni* Steroid biosensor System
CPRG	Chlorophenol Red-β-D-Galactopyranoside
CV	Cyclic Voltammetry
DHT	Dihydrotestosterone
DNA	Deoxyribonucleic Acid
E-screen	Estrogen screen
E1	Estrone
E2	17β-Estradiol
E3	Estriol
EDC	Endocrine Disrupting Compound
EE2	17α-Ethinylestradiol
ELISA	Enzyme-Linked Immunosorbent Assay
ERE	Estrogen Response Element
EU	European Union
GC	Gas Chromatography
GFP	Green Fluorescent Protein

hAR	human Androgen Receptor
hER	human Estrogen Receptor
HRT	Hormone Replacement Therapy
1-OH-PHZ	1-Hydroxyphenazine
4-HPA	4-Hydroxyphenylacetic Acid
3α-HSD/CR	3α-Hydroxysteroid Dehydrogenase/Reductase
IAA	Indole-3-acetic Acid
IBA	Indole-3-butyric Acid
IPTG	Isopropyl-β-D-Thiogalactoside
LB	Luria-Bertani
LC	Liquid Chromatography
LLE	Liquid-Liquid Extraction
MCS	Multiple Cloning Site
MEA	Multielectrode Array
5-MCA	5-Methylphenazine-1-carboxylic Acid
MS	Mass Spectrometry
NAA	α-Naphthalene Acetic Acid
OD	Optical Density
ONPG	Ortho-Nitrophenyl-β-Galactoside
PAA	2-Phenylacetic Acid
PCA	Phenazine-1-carboxylic Acid
PCOS	Polycystic Ovary Syndrome
PCR	Polymerase Chain Reaction
PNEC	Predicted-No-Effect Concentration
RBS	Ribosome Binding Site
RNA	Ribonucleic Acid
SDR	Short-chain Dehydrogenase/Reductases
SOE-PCR	Splicing by Overlap Extension-Polymerase Chain Reaction
SPE	Solid Phase Extraction
SPR	Surface Plasmon Resonance
T	Testosterone

TIRF	Total Internal Reflection Fluorescence
TIRS	Total Internal Reflection Spectroscopy
YAS	Yeast Androgen Screen
YES	Yeast Estrogen Screen

Contents

Scope and outline of the thesis

The scope of this thesis is the design and construction of four different whole-cell bacterial hormone bioreporters. Two proof of principle bioreporters for the detection of auxins, a type of plant hormone, with a relatively easy signaling pathway. Additionally, two bioreporters for the detection of steroid hormones, a type of animal hormone, with a more difficult signaling pathway were also constructed. For each type of hormone two different bioreporters were developed, one with a fluorescent output system and one with an electrochemical readout. The fluorescent output system utilizes the *gfp* gene and the electrochemical readout is based on the production of pyocyanin. The detection of produced pyocyanin of the electrochemical bioreporters is done using a multielectrode array. The integration of the pyocyanin producing bioreporter with the multielectrode array therefore results in a steroid hormone detecting biosensor.

Chapter 1 introduces bioreporters and biosensors, the spread and effects of steroid hormones. The chapter is concluded with a summary of current detection methods for hormones.

In **chapter 2**, all materials and methods used in the construction of the bioreporters and the analysis of the results are listed.

The design and construction of the two whole-cell bacterial auxin bioreporters is outlined in **chapter 3**, followed by the description and discussion of the results obtained using the bioreporters. Both the fluorescent bioreporter and the electrochemical auxin biosensor are functional.

Chapter 4 describes the design and construction of the two whole-cell bacterial steroid hormone bioreporters. The chapter is concluded with the description and discussion of the results obtained using the fluorescent steroid bioreporter. The electrochemical steroid hormone biosensor is not functional.

In **chapter 5** the function of the bioreporters described in chapters 3 and 4 are compared to the function of the traditional methods and existing bioreporters/biosensors described in chapter 1. Followed by a brief look into the ethics of using genetically modified organisms for environmental applications and concluded with the future perspectives.

Chapter 1

General introduction

1.1 Abstract

Bioreporters are microorganisms engineered to produce a signal after detecting the presence of specific molecules, mostly environmental pollutants. When these bioreporters are integrated with a transducer that can measure their signal in a self-contained analytical system they are called biosensors. Currently a large number of bioreporters and biosensors exist that can detect many environmental pollutants.
Steroid sex hormones, a type of animal hormones, have become an environmental pollutant over the last decades due to the increased use of oral contraceptives. Steroid sex hormones have an undeniable detrimental effect on animal live it comes into contact with. Yet, their identification and characterization in environmental samples is rather cumbersome at the moment. The most widely used method to measure the severity of sex steroid pollution in water bodies is mass spectrometry, often in combination with chromatography. This is an expensive and laborious process, therefore alternatives are being researched. Consequently, the development of a steroid hormone bioreporter is described in this work.
Plant hormones on the other hand, more specifically auxins, have become an indispensable addition to fertilizers because of their beneficial effects on plant growth. In addition, the detrimental effects on the environment are negligible as compared to traditional fertilizers, which are very high in nutrients. Simultaneously with the development of the steroid hormone bioreporter, an auxin bioreporter was developed as a proof of principle.

This chapter opens with information on bioreporters and biosensors. This is followed by the spread of steroid hormones in the environment. Next, the effects of both plant and steroid hormones are described, followed by a description of the exisiting methods of detection for environmental hormones. This information forms the background for the development of a whole-cell bacterial hormone bioreporter.

1.2 Sensing the environment

1.2.1 Bioreporters

Engineered bacteria that measure chemicals of environmental concern, so-called bioreporters, have slowly grown in popularity in the last two decades and a half. Their origins can be found in bacterial-based assays, dating back to at least 1973 with the development of the Ames test [4]. This test uses *Salmonella typhimurium* bacteria to test the potential mutagenicity of a chemical. In the years following, researchers have gone one step beyond by genetically engineering bacteria, creating bioreporters designed to measure concentrations of polluting chemicals. One of the first engineered bioreporter was a bioluminescent naphtalene detector [91]. Since then a wide range of genetic components for use as sensor elements and reporter molecules were discovered and developed [1, 6, 32, 184].

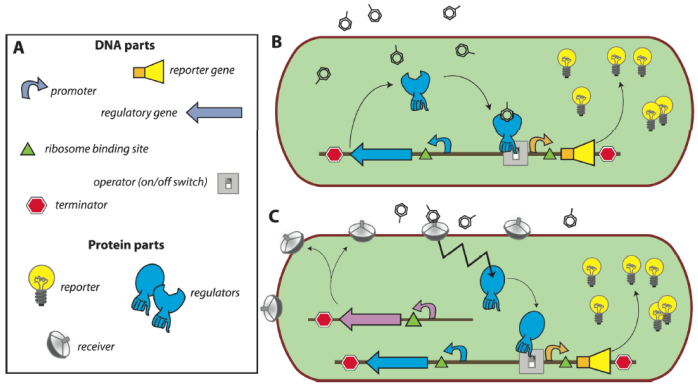


Figure 1.1: Concept of a bacterial bioreporter. (A) Deoxyribonucleic Acid (DNA) parts and their respective proteins necessary for the construction of a bioreporter. (B) Concept of a bioreporter whereby the regulator protein binds the analyte and induces the transcription of the reporter gene. (C) Concept of a bioreporter whereby the analyte is detected by a periplasmic receiver protein that transmits the signal via a signaling cascade to the regulatory protein. Finally, the regulatory protein induces the transcription of the reporter gene. Adapted from Tecon and van der Meer [191].

In essence, bacterial bioreporters are living microorganisms that respond to changes in the environment by producing a specific, measurable signal (Fig.

1.1) [206]. The most commonly used measurable signals or reporter molecules are green fluorescent protein (GFP), bacterial luciferase, firefly luciferase, β-galactosidase, β-glucuronidase, catechol 2,3-dioxygenase and the ice nucleation protein, InaZ [100], each with its own advantages and disadvantages. The choice of reporter molecule is dependent on the intended application of the bioreporter. For example, luciferases are a good choice for highly sensitive applications because of their high quantum yields [191].

The bioreporter can only respond to changes in the environment if it possesses a way to detect these changes. To do this, bioreporters produce regulatory proteins that can bind to specific chemicals and transduce the binding event to transcription of the reporter genes. Since different bacteria species have evolved to adapt to different environments, many regulatory proteins can be found for the detection of specific chemicals (*e.g.*: XylR for the detection of benzene, toluene and xylene; ArsR for the detection of arsenite and antimonite [205]). The existence of these regulatory proteins has led to the construction of bioreporters such as a bioreporter for toluene-based pollutants, using transcriptional activator XylR [218], a phenol-detecting bioreporter by using regulator DmpR [174], bioreporters that detect arsenic in rice, making use of transcriptional repressor ArsR [15] and a bioreporter for the tetracycline group of antibiotics that uses repressor TetR [95].

Traditional analytical methods such as mass spectrometry or chromatography are able to selectively detect concentrations of analytes in the nM range while most published bacterial bioreporters do not perform well at concentrations below 0.1 μM [206]. Despite a lower sensitivity as compared to traditional methods, bioreporters show much promise in monitoring environmental pollutants. Foremost, they are very cost-effective and relatively easy to use because of their small size, rapidity of operation and continuous detection capabilities [29] . In contrast to analytical instruments that measure only the total concentration of the analyte in a sample, bacterial bioreporters detect the fraction which is available to the organism, the bioavailable fraction. Since the bioavailable fraction is the fraction of the total concentration of pollutant that will have an impact on living organisms, the determination of the bioavailable fraction is more interesting than the total concentration since the total concentration might overestimate the risks [67]. The discrepancy between the total and the bioavailable fraction will be larger for analytes with poor aqueous solubility [191].

The results obtained with an ideal bioreporter describe a dose-response relationship between the bioreporter and the analyte that is to be measured. The expression of the reporter molecules should therefore be equivalent to the amount of analyte present.

Limitations of using bioreporters include the need for nutrients to survive and the need for adequate time to express the proteins responsible for the biological

signal [135].

1.2.2 Biosensors

Integrating a bioreporter with a transducer capable of measuring the signals the reporter is emitting, results in a biosensor that can be used as a self-contained analytical measuring system (Fig. 1.2) [29, 135, 191]. Biosensors that use complete unicellular bioreporters as a reporter are referred to as whole-cell biosensors. In whole-cell biosensors, the signal produced by the bacterial bioreporter is typically either optical or electrochemical [29]. Examples of whole-cell biosensors include the use of unicellular algae *Chlorella vulgaris* that produce fluorescence in response to herbicides [207]. By suspending the algae on a rotating support of a homemade flow cell underneath the tip of an optical fibre bundle a fibre-optic biosensor was created (Fig. 1.3). Trögl *et al.* describe an optical biosensor that consists of immobilized *Pseudomonas fluorescens* HK44 cells that produce bioluminescence in the presence of napthalene and salicylate [196].
These examples show that biosensors have much future potential and present an opportunity for cost-effective and easy to use detection of environmental pollutants. Of these pollutants, steroid sex hormones form a growing ecological danger due to their increased spread throughout the environment. The construction of a steroid hormone detecting bioreporter and/or biosensor would greatly increase the ability to quantify the bioavailability of steroid hormones, which is a measure for the effects steroid hormones potentially have on living organisms.

1.2.3 Types of assays performed using bioreporters and biosensors

Several type of measurements can be performed in bioreporter or biosensor assays [191, 205], two of which are shown in figure 1.4. The first type of measurement (Fig. 1.4–A) takes end-point measurements at time t as a function of different analyte concentrations. Analyte concentrations in unknown samples can be quantified by interpolating their induced signal using the calibration curve. In the second type of measurement (Fig. 1.4–B), concentrations C_1 and C_2 are different kind of analyte concentrations whose concentrations are measured in function of time.

The first type of measurements shows the dose-response relationship between the organism and the analyte. The respective figure shows the change in effect on the organism in function of the concentration of analyte. Such curves are typically

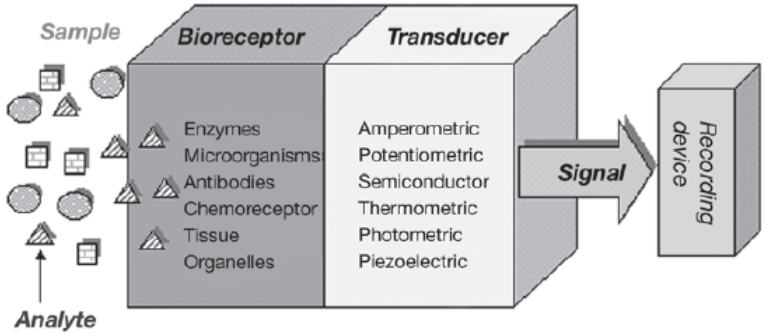


Figure 1.2: Generalized schematic of a biosensor. Sample containing to be detected analyte is brought into contact with the bioreceptor. The binding of analyte and bioreceptor is transduced as a measurable signal to a detector or recording device. Adapted from Newman *et al.* (2006) [133].

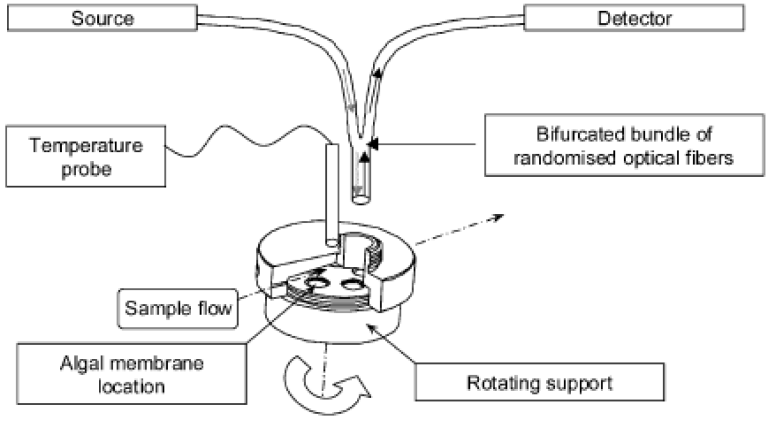


Figure 1.3: Experimental set-up of the algal optical biosensor for continuous toxicity assessment. Adapted from Védrine *et al.* (2003) [207].

sigmoidal and show both a lower plateau, representing the lower treshold concentration needed to exert an effect, and an upper plateau, representing the upper treshold concentration above which effects are no longer seen. A

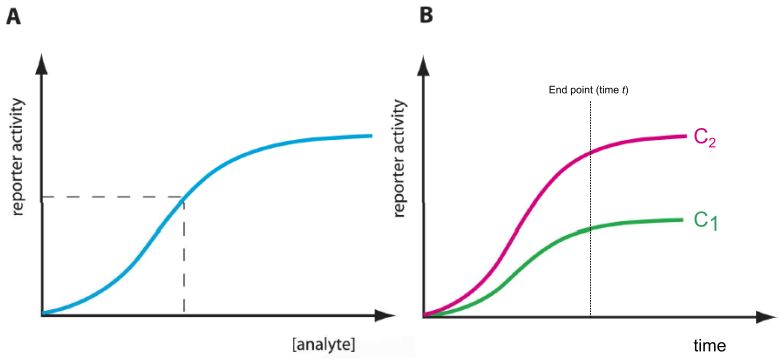


Figure 1.4: Two type of measurements that may be performed in bioreporter or biosensor assays. A. End-point measurements at time t are taken as a function of different analyte concentrations. B. Concentrations C_1 and C_2 are different kind of analyte concentrations whose concentrations are measured in function of time. Adapted from Tecon and van der Meer (2003) [191].

dose-response relationship in the data of the measurements is a prerequisite for an adequate bioreportor or biosensor.

1.3 Spread of steroid sex hormones in the environment

Both natural and synthetic steroid sex hormones (progestogens, androgens and estrogens) are readily released in the environment because of their use as medication [120]. Excreted by humans, spread through municipal wastewater, but also excreted by domesticated animals, spread through manure [13, 94, 175, 229].
Currently, steroid hormones are not considered a priority substance and as such were not assigned an environmental quality standard by the European Union (EU) [51]. This means that no regulations concerning maximum allowed concentrations of steroid hormones in surface waters exists in Europe. Nevertheless, in 1981 the EU prohibited the use of substances having a hormonal action for growth promotion in farm animals (including testosterone, progesterone and 17β-estradiol) [47]. The threat of steroid hormones to public health is therefore acknowledged. Similarly, other endocrine disrupting chemicals, such as bisphenol A (BPA), are being reviewed for possible

identification as priority substance, even though BPA is less potent than steroid hormones [19, 214]. A possible reason for this is the seemingly bigger spread of BPA in surface waters, as compared to steroid hormones. However, steroid hormones are often not detected due to poor limits of detections [19, 108]. Both BPA and steroid hormones are therefore to be found in a large number of surface waters in Europe but only BPA is considered to be a threat, although steroid hormones are more potent than BPA. Evidently, more research is needed to elucidate the possible risks and better detection methods are needed to perform this research.

According to the world urbanization prospects from the United Nations Department of Economic and Social Affairs [201], Belgium is the 15th most urbanized country in the world with 97.5% of the total population living in urban areas. In Europe 73.6% of the total population lives in urban areas and worldwide 54% of all people live in urban areas.

In 2003 the United Nations released their first World Water Development Report. In this report Belgium was ranked last, in the entire world, based upon the quality of its surface water [45, 199]. The European Water Framework Directive is a EU directive which commits EU members to improve both quality and quantity of their water bodies by 2015. Its third implementation report was published in 2012, summarising 2009's data, and shows that 16% of Belgium's natural surface water bodies is of poor quality with respect to chemical pollution (the status of 44% of water bodies is unknown so this number can be far higher than 16%), likewise 73.8% of Belgium's groundwater is of poor quality [48]. Because of this, Belgium (Flanders) still contains the most polluted water bodies in Europe [49].

In general, 74% of Europe's groundwater bodies were reported to be in good chemical status (expectation for 2015: 80%) [50]. Worldwide, improvements in water quality and quantity are being made, partially due to local and overarching directives, but there is still a long way to go [200].

There are a number of reasons for Belgium's bad water quality. Foremost a historical lag in the implementation of sewage treatement and a fragmented accountability for water provision [76]. Brussels' first sewage treatment plant, for example, was built in 2000, up until then all wastewater was released into the river Zenne [7]. Nowadays there are two sewage treatment plants in Brussels and general efforts are being made in Belgium to improve the water quality [46]. Neverthless, the steroid hormone concentrations in water bodies in Flanders, as measured by Dienst Milieurapportering - Afdeling Lucht, Milieu en Communicatie - Vlaamse Milieumaatschappij, are rather high, as shown in table 1.1. The concentrations of estrone (E1) and 17β-estradiol (E2) are relatively high, especially considering concentrations as low as 1 ng/L can have inconvertible effects [94, 209]. The results shown in table 1.1 are from the most recent measurements, performed in 2010. Because the measurements are so

labour-intensive, they are only performed every 5–10 years. Additionally, the samples were only taken from surface waters, there are no plans to examine samples of groundwater. This prevents us from forming a clear image of the scope of steroid hormone pollution in water bodies in Flanders.
Table 1.1 also shows the predicted-no-effect concentrations (PNEC) derived by Caldwell *et al.* to compare measured concentrations of estrogens to concentrations that are expected to elicit health-threatening responses. These PNECs are based upon *in vivo* vitellogenin induction in different fish species. They mentioned values of 6ng/L for estrone, 2ng/L for 17β-estradiol, 60ng/L for estriol (E3) and 0.1ng/L for 17α-ethinylestradiol (EE2) [27]. These values indicate that only low concentrations of exogenous hormones are needed to pose a threat to living organisms. And even though estrogens are poorly soluble in water they can be found in wastewater, surface water, groundwater and even in drinking water [150] in far greater concentrations than the PNEC values [193, 228], even in water bodies in Flanders (Table 1.1). It should be noted that the PNEC values are based upon total concentration, not on the bioavailable concentration. The steroid hormone concentration that actually exerts an effect on the fish is therefore slightly lower.

Table 1.1: Mean and highest measured concentrations of estrone (E1), 17β-estradiol (E2), estriol (E3) and 17α-ethinylestradiol (EE2) measured in surface waters in urban areas spread over Belgium in 2010. Comparison to predicted-no-effect concentrations (PNEC). Measurements performed by Dienst Milieurapportering - Afdeling Lucht, Milieu en Communicatie - Vlaamse Milieumaatschappij.

Hormone	**Mean±SD**	**Highest**	**PNEC**
E1	4.90±9.19 ng/L	56.8 ng/L	6 ng/L
E2	0.78±2.57 ng/L	25 ng/L	2 ng/L
E3	<0.08±0 ng/L	<0.08 ng/L	60 ng/L
EE2	0.08±0.02 ng/L	0.25 ng/L	0.1 ng/L

Endocrine disrupting compounds

Environmental sex steroid hormones can be classified as endocrine disrupting compounds (EDC) because of their endocrine disrupting properties. The World Health Organisation defines an EDC as follows: 'An EDC is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub) populations.' [18]. Various other chemicals are also classified as EDC. These chemicals show hormone-like properties, usually because they bind to

certain hormone receptors [235]. Examples of non-hormone EDC include BPA [127] and alkylphenols [77].
The detection of non-hormone EDC is outside the scope of this work.

1.4 Effects of exogenous hormones

1.4.1 Effects of exogenous steroid hormones

Introduction to steroid hormones

Steroid hormones are hormones that possess the basic structure of four carbon rings known as the steroid nucleus (Fig. 1.5). Cholesterol is a naturally occurring steroid and as such all vertebrate steroid hormones are synthesized from cholesterol. They are primarily synthesized in the gonads, the placenta, the adrenal cortex and the brain. In addition, depending on the function, further modifications may occur elsewhere in the body [136].
Steroid hormones play a vital role in several processes in the human body, including metabolism, salt and water balance, inflammation and sexual development. Besides the naturally occurring steroid hormones, synthetic steroid hormones, like 17α-ethinylestradiol, have been developed to control specific hormonal pathways (*e.g.* 17α-ethinylestradiol prevents follicular development and ovulation) [165]. Hormone effects are mediated by binding to specific receptors [136].
Steroid hormones are divided into five groups based upon the type of receptor to which they bind (Fig. 1.5). The androgens (*e.g.* testosterone (Fig. 1.5–A)), estrogens (*e.g.* 17β-estradiol (Fig. 1.5–B)) and progestogens (*e.g.* progesterone (Fig. 1.5–C)) bind to the androgen receptor, estrogen receptor and progesterone receptor respectively. The glucocorticoids, for example cortisol (Fig. 1.5–D), bind to the glucocorticoid receptor. Mineralocorticoids like aldosterone (Fig. 1.5–E) bind to the cytosolic mineralocorticoid receptor.
Since steroid hormones are active in processes that govern the development and reproduction of animals and humans it is important to minimize the risks associated with faulty hormone signal transduction. Therefore, the amount of exogenous hormones, or analogous molecules, entering the body from outside should be kept to a minimum because they might cause developmental problems [204]. Some of the effects caused by exogenous steroid hormones are listed below.

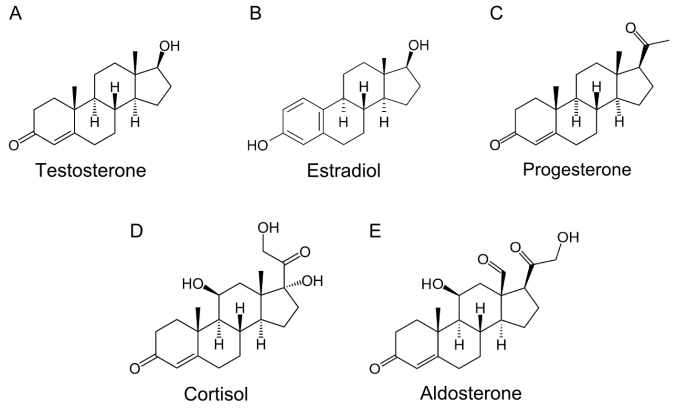


Figure 1.5: Chemical structures of best known members of each steroid hormone group. A. Testosterone (Androgens) B. 17β-estradiol (Estrogens) C. Progesterone (Progestogens) D. Cortisol (Glucocorticoids) E. Aldosterone (Mineralocorticoids).

Glucocorticoids

Corticosterone is a glucocorticoid involved in regulation of energy, immune reactions and stress responses in amphibians, rodents, reptiles and birds, whereas **cortisol** is the primary glucocorticoid in humans which regulates glucose metabolism and inflammation.
In order to study depression and potential antidepressants, a depression model was developed in mice and rats by administering exogenous corticosterone [75, 119]. In this model, subcutaneous corticosterone pellet (1, 2 or 5 mg) implantation causes a constant elevation of plasma corticosterone levels. These elevated corticosterone levels in turn reduce hippocampal cell proliferation and cause the emergence of a "depressed" phenotype [131]. In humans too, a dysregulation of the hypothalamic–pituitary–adrenal axis (in which glucocorticoids are active) has been implicated in major depressions [85, 139].
Dexamethasone and **prednisone** are synthetic glucocorticoids used in the treatment of acute lymphoblastic leukemia. Dexamethasone is preferred because of the higher anti-leukemic activity but both glucocorticoids are also known for their wide range of side effects. Especially dexamethasone induces more neuropsychological, mood and behaviour altering effects [213].
Furthermore both cortisol and corticosterone are known to cause an increase in adipose tissue when chronicly present [149, 160]. And while glucocorticoids are often prescribed as a drug to suppress various allergic, inflammatory and autoimmune disorders, a prolonged use can lead to dire effects including diabetes

[97, 99], osteoporosis [216], hypertensia and many more [147].

Mineralocorticoids

Mineralocorticoids like **aldosterone** and **deoxycorticosterone** are mainly active in the regulation of salt and water balance and in the regulation of blood pressure by acting on the distal tubules of the kidneys. Evidently this means that imbalances in mineralocorticoid levels will disrupt blood pressure which can lead to vascular disease, heart attack and even strokes [101].
In combination with angiotensin II, aldosterone has been known to induce hypertension by acting through the central nervous system [223]. Elevated levels of aldosterone, either through hyperaldosteronism (elevated endogenously) or through exogenous intake, causes damage to cardiovascular tissues [162] leading to hypertension, vascular inflammation [25] and can even lead to heart failure [130].

Progestogens

Progestogens are compounds that maintain pregnancy but they are also present during other phases of the estrous and menstrual cycles. Because of their role in these cycles, progestogens are used in combination with estrogens as a combined oral contraceptive pill. The role of progestogen in this is the inhibition of the follicle development and the inhibition of production of other necessary hormones, this prevents ovulation [161]. Progestogens are taken daily by women all over the world [202]. And because of the nature of the cycles in which progestogens are involved, caution is advised. For example both **progesterone** and **17α-hydroxyprogesterone** were shown to contribute to high blood glucose levels during pregnancy (gestational diabetes mellitus) [9, 159].
Post-menopausal women often undergo hormone replacement therapy (HRT), whereby the hormone levels are artificially boosted to alleviate the symptoms caused by diminshed circulating levels of estrogens and progestogens. Both estrogen- and estrogen-progestogen-HRT exist. Research has shown that estrogen-progestogen-HRT increases breast cancer risk [98, 115, 163] as compared to estrogen only-HRT. But these results may not tell the whole story and further research is undoubtedly needed since more recent studies proved that attributes like race, weight and breast density, a measure for the proportion of the different tissues that make up a woman's breasts, also contribute to the risks [71]. Estrogen-progestogen-HRT might therefore be more suitable for some women than for other.

Androgens

Androgens are described as the compounds that stimulate development and maintenance of male characteristics. This includes the activity of male sex organs and development of male secondary sex characteristics.
Early research has shown that long-term exposure to elevated **testosterone (T)** levels contributes to the development of prostate cancer [60]. However more recent studies minimize this and state that more research is still necessary [31]. Polycystic Ovary Syndrome (PCOS) is a set of symptoms associated with hormone imbalance in women and is the most common endocrine disorder in women, it is also found in female animals [192]. In order to study the disorder, an animal model was developed whereby rats are continuously exposed to **dihydrotestosterone (DHT)** during prepuberty until adult age. Exposed rats will develop typical PCOS [116]. Continuous exposure to either testosterone or DHT during human fetal development has shown that humans can also develop PCOS by androgen excess, although the genetic background also plays an important role [10, 222].
Androgens also promote nitrogen retention and muscle mass [11]. This has led to their use to improve physical performance and misuse of androgens has become a significant problem in sports [63]. Acne, decreased testicular size, decreased sperm count are common effects in men [11]. In women, high doses of androgens can lead to acne, excessive hair growth, excessive vaginal bleeding and masculinization [11]. Furthermore, it is believed that continuous use of androgens can lead to cardiovascular disease [11]. The use of androgens for physical gain is surrounded by secrecy and because of this not all effects are even known.
Finally, androgens also play an important role in governing aggression in animals [3]. And even though hormone-dependent aggression is clearly important in various animals species, there does not appear to be a significant causal link between androgens and human aggression except in rare cases when used in very high doses [8, 152].

Estrogens

As with progestogens, estrogens are compounds of importance in both estrous and menstrual cycles. Estrogens also promote the development of female secondary sex characteristics.
An excess of estrogens has been shown to induce sex reversal in several species of animals [93, 157, 212]. Various species of fish were studied for effects of estrogens in the aquatic environment. It was found that environmental estrogens can impact the reproductive health and persistence of various fish species

[84, 103, 129, 134, 185]. Specifically, full life-cycle exposure of zebrafish (*Danio rerio*) to an environmental relevant concentration of synthetic estrogen **17α-ethinylestradiol (EE2)** resulted in an all-ovary population of fish and a complete inhibition of reproduction [55]. However, zebrafish have the ability to recover and reverse the effects after being removed from the contaminated water and being transferred to clean water [16]. Unfortunately this is not the case for all species of fish. Fathead minnows (*Pimephales promelas*) for example show an increase of vitellogenin in males after exposure to **17β-estradiol (E2)** and **estrone (E1)** [144]. Vitellogenin is an egg yolk precursor protein and is as such mainly produced by females, while male fish can only produce vitellogenin after exposure to exogenous estrogens [141, 169, 178]. Medaka fish (*Oryzias Latipes*) meet the same fate when exposed to E2 [171]. For this reason both medaka and fathead minnows are often used to measure estrogenicity in waters [126, 144].
Amphibians display genetic sex determination but this determination can be influenced by external factors. One of these external factors is exogenous estrogens. The African clawed frog *Xenopus laevis* for example will produce 100% females when larvae are exposed to exogenous estrogens, regardless of genetic makeup [69].
The sex of turtles is largely determined by hatching temperatures of the eggs. But even at male-producing temperatures (26°C), feminization could be induced through exposure to E2 [34]. Adult male turtles will also produce high levels of vitellogenin after E2 injections [143].
In humans, elevated estrogen levels have been known to induce tumor growth [20, 118, 177, 224] in both women and men [195]. Moreover, high estrogen levels are also linked to thrombosis and thromboembolism [42, 190].

1.4.2 Effects of exogenous auxins

Introduction to auxins

Plant hormones or phytohormones are signal molecules that regulate cellular processes mostly related to plant growth. Phytohormones are categorized into several groups based upon their chemical structures including abscisic acid, auxins, cytokinins, ethylene and gibberellins [168, 217]. Of these, auxins were the first major plant hormones to be discovered. They play an important role in many aspects of plant growth and development [168, 231]. All auxins are compounds containing aromatic rings and a carboxylic acid group. The most abundant auxin is indole-3-acetic acid (IAA) (Fig. 1.6–A). Besides IAA, three additional natural occurring auxins exist: *i.e.* 2-phenylacetic acid (PAA) (Fig. 1.6–B), indole-3-butyric acid (IBA) (Fig. 1.6–C) and 4-chloroindole-3-acetic

acid (4-Cl-IAA) (Fig. 1.6–D). These are all synthesised from indole, which itself is derived from chorismic acid inside the chloroplasts [117]. Additionally, several synthetic auxins have been synthesized for agricultural purposes. For example α-naphthalene acetic acid (NAA) (Fig. 1.6–E) is often used to stimulate root growth [43, 225].

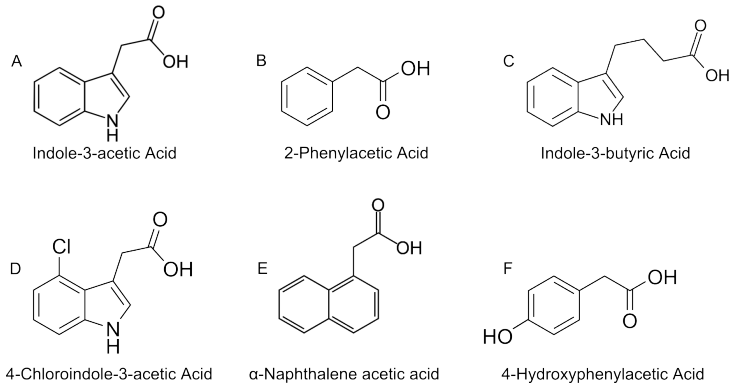


Figure 1.6: Chemical structures of: A. Indole-3-acetic Acid (IAA), B. 2-Phenylacetic Acid (PAA), C. Indole-3-butyric Acid (IBA), D. 4-Chloroindole-3-acetic Acid (4-Cl-IAA), E. α-Naphthalene acetic acid (NAA), F. 4-Hydroxyphenylacetic acid (4-HPA)

Effects of exogenous auxins

Since auxins are active in processes that govern the development and growth of plants, they are an interesting candidate for detection. Some of the effects caused by exogenous auxins are listed below.

Exogenously added **IAA** has been shown to increase shoot and root elongation [24, 87, 189]. However, if the concentration of added IAA is too high (>5 μg/mL [24]), a decrease in plant growth can be seen [146]. The effects of too high concentrations of IAA can be mitigated by using bacteria to introduce and regulate the concentration of IAA in the rhizosphere [65].

Exogenous **PAA** induces organogenesis [81], improves bud elongation [38, 72] and has a positive influence on clonal propagation [61].

The addition of **IBA** to plants has proven to induce the growth of both new regular roots [86, 173] and adventitious roots [112]. Adventitious roots are formed in unusual places on a plant, usually a stem or a leaf. They are of

interest in horticulture and agronomy because of the many plant species that are difficult to root.

Exogenously added **4-Cl-IAA** induces root formation [88] and an increased elongation and growth [87]. Moreover, its beneficial effects can cancel the deleterious effects of cadmium stress [124].

Synthetic auxin **NAA** has positive effects on adventitious root development [225]. Both fruits [142] and plants [198] increase in size after being exposed to NAA. Additionally, exogenously added NAA increases the expression of antioxidant enzymes [43].

1.5 Detection of environmental hormones

In order to sufficiently gauge the severity of the situation, an efficient method of detection for environmental steroid and plant hormones is needed. An overview of currently used methods was published by Maser and Xiong [121]. In this paper the distinction is made between all the methods that measure the total concentration of steroid and plant hormones directly and specifically, and all the methods that determine the concentration of hormones based upon the biological effect caused on the sensing system, *i.e.* methods that determine the bioavailable fraction.

1.5.1 What makes a good detection method?

The quality of a detection method can be determined based on a number of criteria. In particular for methods to detect steroid hormones, the following criteria are important [54] :

- Short sample preparation time
- Short measurement preparation time
- Short measurement time
- Short analysis time
- Low limit of detection
- Specific for multiple steroid hormones

- Inexpensive

1.5.2 Direct measurement of concentration

Chromatography and mass spectrometry

The most used techniques to directly measure steroid hormones are liquid or gas chromatography (LC/GC) and mass spectrometry (MS) [5, 78, 109, 211]. MS is an analytical technique used to identify molecules. A mass spectrometer consists of three parts: an ion source, a mass analyzer and a detector. Samples are introduced in the ion source where they are ionized. This may cause the molecules to break down in smaller parts. Afterwards they are introduced in the mass analyzer where the molecules will be separated based upon their mass-to-charge ratio. And finally the detector will measure the different molecules in the sample. The result of a MS procedure is pictured as a spectrum (Fig. 1.7–A).
Chromatography techniques are used to separate mixtures of molecules. The mixture is usually dissolved in a gas or fluid which is called the mobile phase. The mobile phase will carry the mixture through the stationary phase. Because the different molecules in the mixture move at different speeds through the stationary phase, the molecules are separated. If the mobile phase is a gas, the technique is referred to as gas chromatography (Fig. 1.7–B) and if it is a liquid, the technique is called liquid chromatography (Fig. 1.7–C).

Chromatography and MS are often used in combination. These two methods offer precise and highly specific concentration measurements of water samples. But in order to use either chromatography or mass spectrometry, the samples need to be extensively prepared beforehand.
Samples taken from large bodies of water are always a complex mixture of molecules. This mixture of molecules surrounding the analyte of interest is generally called a matrix. Whenever samples inside of a matrix are analysed, certain matrix effects can occur whereby the matrix influences the analysis due to its intrinsic physical or chemical properties (*e.g.* ionic strength of the solution). It is therefore important to remove the analyte of interest from the matrix to reduce the impact of the matrix effects [153].
The process of removing analytes from their matrix is called extraction, commonly performed by solid phase extraction (SPE) or liquid-liquid extraction (LLE). SPE is a separation process whereby dissolved compounds are separated from other compounds in the mixture by passing the mixture (known as the mobile phase) through a solid (known as the stationary phase) (Fig. 1.8–Top) [232]. Either the analyte of interest or the undesired analyte is retained in the stationary phase, the part of the mixture that passed through the stationary

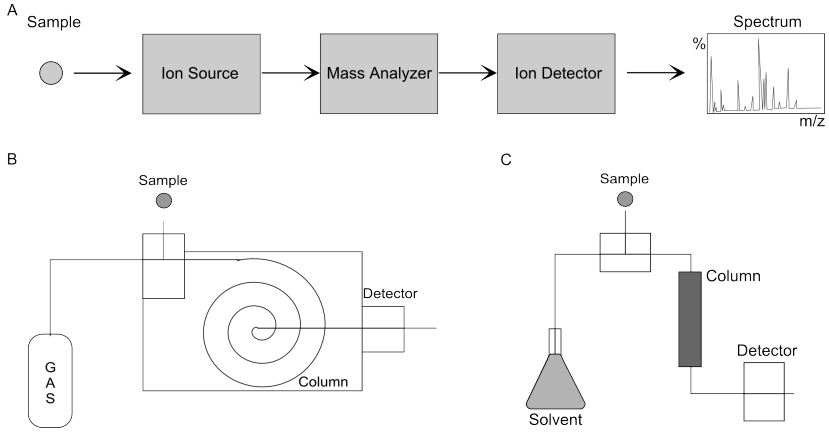


Figure 1.7: Schematical overview of A. Mass Spectrometry: Sample is introduced into ion source where it is ionized, causing the molecule to break up into smaller parts, these parts are brought into the mass analyzer where the molecules are separated based upon their mass-to-charge ratio. Finally the detector measures the arrival of the molecules of the sample. The result is shown as a spectrum. B. Gas Chromatography: The sample is introduced into a column in which gas (mobile phase) flows, the gas carries the sample through the column (stationary phase). The different molecules in the sample will move at different speeds through the column, causing them to separate. The detector measures the arrival of all the different molecules. C. Liquid Chromatography: The sample is introduced into a column in which a fluid (mobile phase) flows, the fluid carries the sample through the column (stationary phase). The different molecules in the sample will move at different speeds through the column, causing them to separate. The detector measures the arrival of all the different molecules.

phase will then be kept or discarded, depending on whether it contains the analyte of interest or not. LLE is a method to separate compounds based upon their relative solubility in two different immiscible liquids (Fig. 1.8–Bottom) [188]. The analyte of interest should be more soluble in the second liquid than in the original liquid. In the case of steroid hormones, chloroform is often used for its extraction from water samples [40]. Both liquid-liquid extraction and solid phase extraction are used for the extraction of steroid hormones from water samples [5, 40, 78, 110]. Only after performing the extraction can the analyte be further analyzed by chromatography and/or MS. The result is a highly specific and sensitive measurement ($0.01<T<0.6$ ng/L, $0.01<P<0.23$ ng/L, $0.01<E1<0.04$ ng/L [211], 0.1-2.4 ng/L [78], 2-500 ng/L [109]) depending on used chromatography and spectroscopy techniques, obtained only hours after

sample collection [78, 109, 211].

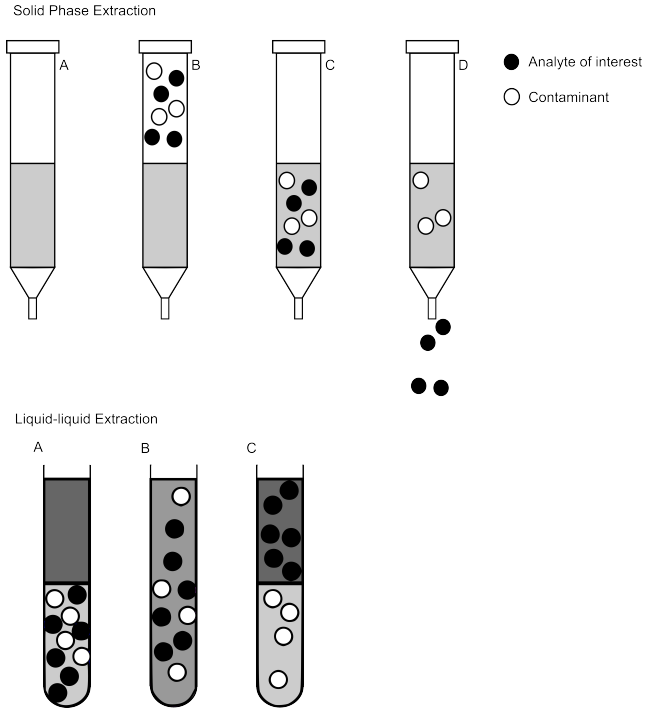


Figure 1.8: Top: Solid Phase Extraction (SPE). A. Empty column, grey are represents stationary phase, B. Sample inserted, C. Sample on stationary phase, D. Contaminants are retained and analyte of interest passes through. Bottom: Liquid-liquid Extraction (LLE). A.Sample in solution, second liquid added, B. Mixing liquids by shaking, C. Liquids unmix because they are immiscible, analyte of interest is in solution in the second liquid and can be removed.

Both soil auxins and endogenous auxins can be detected using chromatography and/or MS after extraction [106, 137]. The sensitivity ranges between 20 ng/L [111] and 2 μg/L [102].
Due to the high specificity, chromatography and/or MS is suitable for the detection of a variety of steroid or plant hormones. The greatest disadvantage is the labour and time intensive preparation needed for an accurate measurement. Sampling aside, which can take up to two days, the preparation time can run up to several hours, mostly spend on extraction and drying. Measuring the samples can take up as much as an hour [5, 109, 211].

Immunoassays

Antibodies are glycoproteins that are part of the immune system. They identify and neutralize foreign objects entering the body whereby each antibody recognizes a specific antigen unique to its target [80]. The specific nature of an antibody makes it eligible for use as a sensor. Sensors using antibodies to detect compounds are called immunosensors [113]. Since antibodies do not have a way of transducing the signal, a supplementary method is needed to transduce the signal. These supplementary methods can be divided into groups based upon the physical property which they employ to transduce the signal. The two largest groups are the electrochemical and the optical sensors.
Electrochemical immunosensors are always built to be used with an electrode that measures potential changes, current flows or conductivity changes. Electrochemical immunosensors are usually used in a competition assay whereby the analyte of interest competes with another ligand for binding to the antibodies. In general, the competing ligand is the same as the analyte of interest but labelled with an enzyme that can reduce or oxidize molecules (*e.g.* peroxidases). The amount of labelled analyte bound to the antibodies is a measure for the amount of unlabelled analyte in the sample and the former amount can be determined thanks to its enzymatic function which causes a reduction or oxidation. This is measurable using an electrode that can determine the amount of analyte in the sample (Fig. 1.9–Top).
Examples of electrochemical immunosensors used to detect steroid hormones include a competition assay between estradiol and peroxidase-labelled estradiol [138], between estradiol and bovine serum albumin (BSA)-labelled estradiol in a complex scaffold [107] and between testosterone and peroxidase-labelled testosterone by use of magnetic beads to immobilize the antibodies [41]. The detection limit for electrochemical immunosensors were reported to be as low as 1.7 ng/L [41].

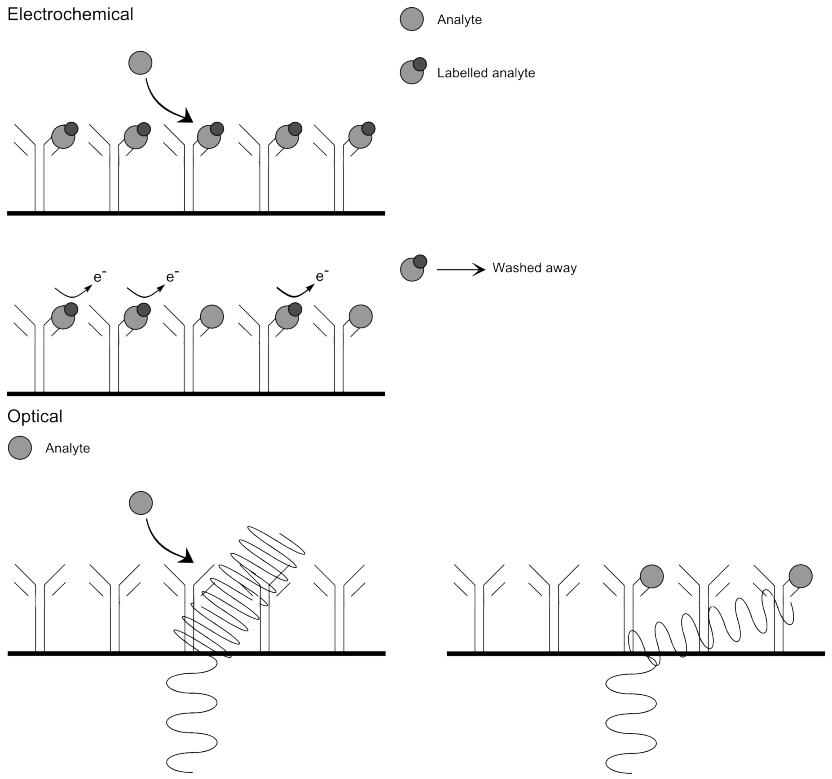


Figure 1.9: Top: Electrochemical immunosensor: Antibodies attached to a surface bound to labelled analyte. Unlabelled analyte is introduced and will displace the labelled analyte from the antibodies. The displaced labelled analytes are washed away and a current is measured. This current is a measure for the amount of bound labelled analyte which is a measure for the amount of unlabelled analyte. Bottom: Optical immunosensor: Antibodies are attached to a surface. Analyte is introduced and will bind to the antibodies. Light shone on the surface will behave differently dependent on the amount of analyte bound to the antibodies.

There also exists a number of auxin electrochemical immunosensors [227, 233] that can detect concentrations from 50 ng/L [186].
Optical immunosensors are based upon the physical properties of light reflected of a surface. These physical properties (*i.e.* adsorption, fluorescence, luminescence, scattering or refractive index) can differ depending on whether or not certain molecules are present on the surface. In an optical immunosensor, light is shone

upon a surface on which antibodies are attached. A sample containing the analyte that binds the antibodies, is released on the surface. The binding of the analyte to the antibodies influences the physical interaction of the light with the surface and a change in physical properties will be measured. This change is a measure of the amount of analyte present in the sample (Fig. 1.9–Bottom). Popular applications of these principles are surface plasmon resonance (SPR) [166] and total internal reflection fluorescence (TIRF) [14, 44].
These experimental designs were applied to the detection of steroid hormones, mostly in combination with SPR with a detection limit as low as 50 ng/L [66, 230].

Immunosensors are made both with monoclonal and polyclonal antibodies. Each have their advantages and disadvantages for use as a steroid detection method. Monoclonal antibodies generally are expensive to make, require advanced technology for which extensive training is needed and can take a long time to produce [80]. A large amount of monoclonal antibodies can be produced at once but these antibodies can be too specific and therefore can only detect one type of molecule. Polyclonal antibodies are inexpensive to make, require less advanced technology [80]. Therefore no advanced training is needed and antibodies can be produced in a short time. The produced polyclonal antibodies are non-specific to the point that background noise can occur and variability between batches can exist. Either kind of antibodies has it advantages and disadvantages which means that for use as steroid hormone detector choices have to be made. Either monoclonal antibodies are used to construct an expensive, labour-intensive, but highly specific detector that needs a long time to prepare. Or polyclonal antibodies are used to produce an inexpensive, easy to manipulate detector with short preparation times but highly variable and unspecific [80]. Neither kind of antibody grants a complete solution.
While antibodies take a long time to prepare, immunosensors have to be build up as well (*e.g.*: attaching antibodies to a surface), this generally requires more than three hours [107, 138, 230]. The measurements of the samples only take up 30 minutes. This lengthy preparation time and the need for antibodies make immunosensors rather expensive [107, 138, 230]. The data acquired during measurements is relatively easy to interpret. Voltammograms for example, easily show the presence of interesting analytes. Optical immunosensors are even label free, a label molecule is not required for detection of the analyte. The biggest downside of immunosensors is the need for antibodies.

1.5.3 Determination of concentration based upon biological effect

All aforementioned methods are based upon detection of the chemical structures of interesting analytes. Because of the great diversity of steroid hormones this direct approach becomes impractical and expensive. It might be more interesting to design a method that can identify these substances by their hormonal activity rather than by their structure. Such a detection method measures the biological effect of non-steroid molecules as compared to that of steroid hormones. A range of methods that can assess the potential risk without structural knowledge, based on biological effects has already been devised. The most important of these methods are vitellogenin assays, the estrogen screen (E-screen), yeast estrogen/androgen screen (YES/YAS) and the *Comamonas testosteroni* steroid biosensor system (COSS).
For the detection of auxins, few methods based upon biological effects have been developed at this time. The most important contribution is the DII-VENUS.

Vitellogenin assay

Vitellogenin is a glycolipoprotein and functions as an egg yolk precursor protein. During egg development, vitellogenin is cleaved into the lipo- and phosphoproteins that make up most of the protein content of egg yolk. In normal circumstances vitellogenin is produced by the females of nearly all oviparous species, like birds, most fish, amphibians and reptiles. But in the presence of estrogens or estrogenic EDC, male fish can also produce vitellogenin in a dose dependent manner. That is why several species of fish were already used to assess the estrogenicity (*i.e.*: capacity of compounds to act like an estrogen) of surface waters by placing them downstream of a suspected estrogen source [36, 57, 144, 148]. Blood of these fish is harvested via tail severance [36] or cardiac puncture [144]. The harvested blood is centrifuged and afterwards analyzed using ELISA with, as a positive control, vitellogenin from fathead minnows exposed to E2 purified using anion-exchange chromatography.
The use of fish or amphibians, to determine the estrogenicity of a surface water, can give a clear image over a period of time (as low as ng/L concentrations) as opposed to the static time point by which one is limited to using analytical analysis. Vitellogenin assays also take into account solubility, temperature and other physical properties for an accurate depiction of the estrogenicity [57]. However, the use of these fish is not suitable for the determination of a specific estrogenic compound since all estrogenic compounds will exert an influence on the fish. For the detection of a specific estrogen or estrogenic compound at any given time, analytical analysis will be more accurate and sensitive but also far

more specific. Analytical analysis will also be faster as the incubation period for vitellogenin analysis can be up to one [57] or even three weeks [148]. In addition, vitellogenin analysis occurs after harvesting blood via tail severance or cardiac puncture. This is both time consuming, labour-intensive and requires the sacrifice of living animals.

The estrogen screen

The E-screen is a quantitative assay based upon the proliferative effect of estrogens on MCF-7 cells. MCF-7 is a breast cancer cell line and one of the first mammary cell lines that could be kept in culture for a long period of time. Cells of this line show a proliferative response to estrogens. The E-screen uses this property to measure the estrogenicity of a compound by comparing the proliferative response of the compound to the response of E2 (positive control) and of a sample without estrogens (negative control) [182]. A number of compounds with estrogenic properties were found in this way, such as alkylphenols and phtalates [181, 182].
The E-screen can detect estrogenic compounds with high sensitivity (E2 can be measured at as low as 8 ng/L) but the method is time consuming and labour-intensive. The cells require full cell culturing and sample incubation time has to be up to six days for optimal results [145]. While this method can be used to measure estrogenicity as compared to E2, it fails to measure actual concentrations of estrogens or steroid hormones in the sample and since it measures estrogenicity, the method is not very specific [182].

Yeast estrogen/androgen screen

Yeast is a single cell eukaryotic organism and is therefore interesting for scientific research since it can easily be cultured, has a short generation time and can be used to express eukaryotic proteins. This last property is the basis of the yeast estrogen/androgen screen (YES/YAS) [58, 164]. These two methods were developed by integrating the gene coding for the human estrogen receptor (hER) or the human androgen receptor (hAR) in the yeast genome. After binding to an estrogen, hER wil bind to the estrogen response element (ERE) and induce the expression of the downstream gene. hAR works similarly by binding to the androgen response element (ARE) in the presence of androgens. Plasmids carrying ERE or ARE followed by *lacZ* ensure signal transduction whereby the presence of estrogens or androgens will induce the expression of β-galactosidase. Production of β-galactosidase can be observed by adding a chromogenic substrate (*e.g.*: chlorophenol red-β-D-galactopyranoside (CPRG))

which will be converted by β-galactosidase and hereby change colour.
Because of their short generation time and ability to express eukaryotic proteins, YES and YAS can contribute greatly to the detection of steroid hormones. The technique is sensitive (E2 detection at 3 ng/L), reproducible [164] and is specific to estrogens and estrogenic compounds. Sample preparation and analysis can happen relatively quickly. Measurement and measurement preparation on the other hand are rather labour-intensive because of the use of the *lacZ* reporter system and typically take 4 days to a week [58, 164].

Comamonas testosteroni steroid biosensor

Xiong *et al.* constructed a *Comamonas testosteroni* (*C. testosteroni*) mutant strain CT-GFP5-1 that can be used as a fluorescence based biosensor system for the detection of steroids [219]. The system is based on the steroid biodegradation mechanism of *C. testosteroni* and was constructed by placing the *gfp* gene into the chromosome of *C. testosteroni*, downstream of a steroid responsive promoter. This resulted in a whole-cell bioreporter that can detect T concentrations between 57 and 450 μg/L and E2 concentrations between 1.6 and 12.8 μg/L. The experimental COSS setup was improved upon by developing a cell-free system that only uses the cytosol of the mutant. The cell-free system can detect T concentrations between 28 and 219 ng/L and E2 concentrations between 29 pg/L and 430 pg/L.
The preparation time needed for the cell-based system is short and easy, for the determination of the concentration of steroids in a sample using the cell-based system only an overnight culture is needed. To determine a steroid concentration in a sample using the cell-free system the cytoplasm of CT-GFP5-1 mutants needs to be isolated and fixed onto a 96-well plate, this requires more time and effort. The measurements themselves take 30 minutes for both the cell-based and cell-free systems. The specificity of CT-GFP5-1 bacteria is not sufficiently characterized, however it is defined as a steroid bioreporter and can detect cholesterol as well. This bioreporter is therefore not specific to steroid hormones alone.

DII-VENUS for the detection of auxins

DII-VENUS is an Aux/IAA-based auxin signalling sensor that was engineered in the model plant *Arabidopsis thaliana*. DII-VENUS consists of the fast maturing YFP VENUS fused in-frame to the Aux/IAA auxin-interaction domain (domain DII), controlled by a constitutive promoter [26]. The result of this fusion is that DII-VENUS will be degraded dose-dependently in the presence of IAA. The DII-VENUS sensor can therefore be used to verify the distribution of IAA in

Arabidopsis and can detect concentrations of IAA as low as 175 ng/L. However, while DII-VENUS is able to detect the transport of endogenous auxins in the plant, it is not a suitable sensor for the determination of exogenous auxin concentrations since it only detects the auxins the plant has taken up. Moreover, the sensor requires up to five days preparation time.

1.5.4 Comparative table

Table 1.2: Table comparing the properties of each method.

Method	**Preparation time**	**Measurement time**	**Sensitivity**	**Specificity**
GC/LC-MS	1–2 h	1–2 h	10 pg/L	specific molecules
Immunoassays	>3 h	30 min	50 ng/L	specific molecules
Vitellogenin assay	1 day	1–3 weeks	ng/L	estrogenic compounds
E-screen	1 day	6 days	8 ng/L	estrogenic compounds
YES/YAS	1 day	1–3 days	3–8 ng/L	estrogenic compounds
COSS (cell-based)	12 h	30 min	1.6 μg/L	steroids
COSS (cell-free)	14 h	30 min	29 pg/L	steroids
DII-VENUS	5 days	2 h	175 ng/L	IAA

1.6 Conclusions

This introductory chapter shows that both humans ands animals excrete steroid hormones from their bodies which end up in the environment through municipal wastewater [120, 229]. These steroid hormones, estrogens in particular, can have detrimental effects on humans and animals [20, 42, 84, 103, 129, 134, 185, 224]. And while traditional methods exist that can detect the hormones in our waters, they require extensive time and labour to prepare the samples to be measured. A novel cost-effective, easy to use, *in situ* sensor could be a screening tool to rapidly determine possible steroid hormone pollutions in nature. Such a screening tool might be provided by constructing a whole-cell bioreporter or biosensor.

The next chapters describe the design and construction of a GFP-producing steroid hormone bioreporter to offer a cost-effective, easy to use substitute. Likewise, an electrochemical biosensor was constructed based on the results of the GFP-producing bioreporter. The electrochemical biosensor is based on the novel pyocyanin reporter system. The signal produced by this unique reporter system can be directly transduced to a computer thanks to the redox properties of pyocyanin. A pyocyanin electrochemical steroid hormone biosensor should therefore offer an efficient fast-acting biosensor that might be adapted to *in situ* applications.

This work also describes a GFP-producing auxin bioreporter and an electrochemical auxin biosensor. These two were constructed as a proof of principle. The mechanism to detect auxins is significantly less complex than that to detect steroid hormones. The proof of principles were therefore used to determine the feasibility of the proposed steroid hormone reporters.

Chapter 2

Materials and Methods

2.1 Bacterial strains and growth conditions

All strains and plasmids used in this work are listed in table 2.2. All strains were grown with aeration at 37°C in Luria-Bertani (LB) medium or on LB plates containing 15 g/L agar (Invitrogen) [167].
Standard protocols were used for molecular cloning [167]. Restriction enzymes were purchased from Roche and used according to the manufacturer's instructions. Cloning steps were performed using *E. coli* TOP10. All primers used for cloning in this work are listed in table 2.4.

ONPG-buffer

- 50 mM Phosphate buffer pH 7
- 10 mM β-mercaptoethanol
- 0.1% Triton X100
- 0.1% Sodium Lauryl Sarcosine
- 1 mM Na_2EDTA

2.1.1 Plasmid construction

Construction of pCMPG10652

Bacteria transformed with plasmid pCMPG10652 produce GFP when exposed to auxins. The plasmid is therefore used to construct a fluorescent auxin bioreporter (Section 3.3.2).
The DNA sequence containing promoter P_A, *hpaA* and promoter P_{BC} of pRA_2 [155] was amplified using primers PRO6977 and PRO7618. This PCR product was then cloned into pFPV25 [203], using EcoRI and XbaI, resulting in pCMPG10652.
pCMPG10652 was maintained with 100 μg/mL ampicillin.

Construction of pCMPG10653

Bacteria transformed with plasmid pCMPG10653 produce pyocyanin when exposed to auxins. The plasmid is therefore used to construct an electrochemical auxin bioreporter and biosensor (Section 3.3.3).
The DNA sequence containing promoter P_A, *hpaA* and promoter P_{BC} of pRA_2 [155] was amplified using primers PRO7995 and PRO8160. This PCR product was then cloned into pUCP-MS [122], using EcoRI, resulting in pCMPG10653.
pCMPG10653 was maintained with 10 μg/mL tetracyclin.

Plasmid pCMPG10654

Bacteria transformed with plasmid pCMPG10654 produce GFP when exposed to steroid hormones. The plasmid is therefore used to construct a fluorescent steroid hormone bioreporter (Section 4.3.1).
The soil bacterium *C. testosteroni* contains a steroid hormone responsive sequence in its genome. This sequence contains a constitutive promoter flanked by two operators and the *repA* gene, encoding repressor RepA. RepA binds to the two operators in the absence of steroid hormones, blocking the promoter and repressing the expression of the downstream gene. In the presence of steroid hormones RepA will bind those and release the operators, freeing the promoter and allowing for the expression of the downstream gene. For this reason, the DNA sequence containing the two operator sites of the *C. testosteroni* genome was amplified using primers PRO7047 and S&P0058. The DNA sequence containing the *gfp* gene of pFPV25 [203] was amplified using primers S&P0059 and S&P0060. Both PCR products possessed an overhang which allowed for them to be used as primers in a SOE-PCR reaction [70] where both products

were spliced together whereby the *gfp* gene was placed downstream of the two operator sites. This final product was ultimately cloned into pFAJ5301 [210], using EcoRI and XbaI, resulting in pCMPG10654.
pCMPG10654 was maintained with 150 μg/mL erythromycin.

Plasmid pCMPG10658

Plasmid pCMPG10658 contains the *repA* gene, encoding the repressor RepA, downstream of a P_{BAD} promoter (Sections 4.3.1).
The DNA sequence containing the *repA* gene of the *C. testosteroni* genome was amplified using primers S&P0061 and S&P0062. This PCR product was then cloned into pBAD322K [35], using XbaI and HindIII, resulting in pCMPG10658.
pCMPG10658 was maintained with 50 μg/mL kanamycin.

Plasmid pCMPG10659

Plasmid pCMPG10659 contains the *repA* gene, encoding the repressor RepA, downstream of a strong constitutive promoter (Section 4.3.1).
The DNA sequence containing the *repA* gene of the *C. testosteroni* genome was amplified using primers S&P00137 and S&P00141. The DNA sequence containing the strong constitutive promoter J23100 and the strong RBS B0034 was amplified using primers S&P00142 and S&P00143. Both PCR products possessed an overhang which allowed for them to be used as primers in a SOE-PCR reaction [70] where both products were spliced together whereby the *repA* gene was placed downstream of the promoter and RBS combination. This final product was ultimately cloned into pUC19, using XbaI and HindIII, resulting in pCMPG10659.
pCMPG10659 was maintained with 100 μg/mL ampicillin.

Plasmid pCMPG10660

Plasmid pCMPG10660 contains the *repA* gene, encoding the repressor RepA, downstream of a weak constitutive promoter (Section 4.3.1).
The DNA sequence containing the *repA* gene of the *C. testosteroni* genome was amplified using primers S&P00137 and S&P00138. The DNA sequence containing the weak constitutive promoter J23114 and the strong RBS B0034 was amplified using primers S&P00139 and S&P00140. Both PCR products possessed an overhang which allowed for them to be used as primers in a SOE-PCR reaction [70] where both products were spliced together whereby the *repA* gene was placed downstream of the promoter and RBS combination. This final

product was ultimately cloned into pUC19, using XbaI and HindIII, resulting in pCMPG10660.
pCMPG10660 was maintained with 100 μg/mL ampicillin.

Plasmid pCMPG10661

Plasmid pCMPG10661 contains the *repA* gene, encoding the repressor RepA, downstream of a weak constitutive promoter (Section 4.3.1).
The DNA sequence containing the *repA* gene of the *C. testosteroni* genome was amplified using primers S&P00137 and S&P00144. The DNA sequence containing the strong constitutive promoter J23104 and the medium RBS B0032 was amplified using primers S&P00145 and S&P00146. Both PCR products possessed an overhang which allowed for them to be used as primers in a SOE-PCR reaction [70] where both products were spliced together whereby the *repA* gene was placed downstream of the promoter and RBS combination. This final product was ultimately cloned into pUC19, using XbaI and HindIII, resulting in pCMPG10661.
pCMPG10661 was maintained with 100 μg/mL ampicillin.

Plasmid pCMPG10662

Plasmid pCMPG10662 contains the *repA* gene, encoding the repressor RepA, downstream of a strong constitutive promoter (Section 4.3.1).
The DNA sequence containing the *repA* gene of the *C. testosteroni* genome was amplified using primers S&P00137 and S&P00144. The DNA sequence containing the medium constitutive promoter J23110 and the medium RBS B0031 was amplified using primers S&P00146 and S&P00147. Both PCR products possessed an overhang which allowed for them to be used as primers in a SOE-PCR reaction [70] where both products were spliced together whereby the *repA* gene was placed downstream of the promoter and RBS combination. This final product was ultimately cloned into pUC19, using XbaI and HindIII, resulting in pCMPG10662.
pCMPG10662 was maintained with 100 μg/mL ampicillin.

Plasmid pCMPG10663

Plasmid pCMPG10660 contains the *repA* gene, encoding the repressor RepA, downstream of a medium constitutive promoter (Section 4.3.1).
The DNA sequence containing the *repA* gene of the *C. testosteroni* genome was amplified using primers S&P00137 and S&P00148. The DNA sequence

containing the medium constitutive promoter J23110 and the weak RBS B0032 was amplified using primers S&P00147 and S&P00149. Both PCR products possessed an overhang which allowed for them to be used as primers in a SOE-PCR reaction [70] where both products were spliced together whereby the *repA* gene was placed downstream of the promoter and RBS combination. This final product was ultimately cloned into pUC19, using XbaI and HindIII, resulting in pCMPG10663.
pCMPG10663 was maintained with 100 μg/mL ampicillin.

Plasmids pCMPG10657 and pCMPG10664

Plasmid pCMPG10664 contains the two operator sites of the *C. testosteroni* genome both upstream of the *phzM* and the *phzS* gene and should therefore produce pyocyanin in the presence of steroid hormones (Section 4.3.2).
The DNA sequence containing the two operator sites of the *C. testosteroni* genome was amplified using primers S&P00421 and S&P00422. The DNA sequence containing the genes *phzM* and *phzS* of pUCP-MS [122] was amplified using primers S&P00423 and S&P00424. Both PCR products possessed an overhang which allowed for them to be used as primers in a SOE-PCR reaction [70] where both products were spliced together whereby the *phzM* and *phzS* genes were placed downstream of the two operators. This splice product was cloned into plasmid pBBR1MCS-2 using HindIII and EcoRI, resulting in plasmid **pCMPG10657** (Fig. 2.1).
The DNA sequence containing the operator sites and the gene *phzM* of the former PCR product was amplified using primers S&P00421 and S&P00636. This PCR product was cloned into plasmid pBBR1MCS-2 using BamHI and EcoRI, resulting in plasmid **pTEMP1** (Fig. 2.1).
The DNA sequence containing the operator sites of the *C. testosteroni* genome was amplified using primers S&P00637 and S&P00639. The DNA sequence containing the gene *phzS* of pUCP-MS [122] was amplified using primers S&P00638 and S&P00424. Both PCR products possessed an overhang which allowed for them to be used as primers in a SOE-PCR reaction [70] where both products were spliced together whereby the *phzS* gene was placed downstream of the newly amplified operators. This splice product was cloned in plasmid pTEMP1 using HindIII and EcoRI, resulting in plasmid **pTEMP2** (Fig. 2.1).
pTEMP2 also failed to produce pyocyanin, possibly because of how the two instances of the operators were orientated. For this reason, one of the operators and gene combinations was amplified using primers S&P00674 and S&P00675. This PCR product was cloned in pBBR1MCS-2 in the opposite orientation, using EcoRI and HindIII to give **pCMPG10664** (Fig. 2.1).
pCMPG10664 was maintained with 50 μg/mL kanamycin.

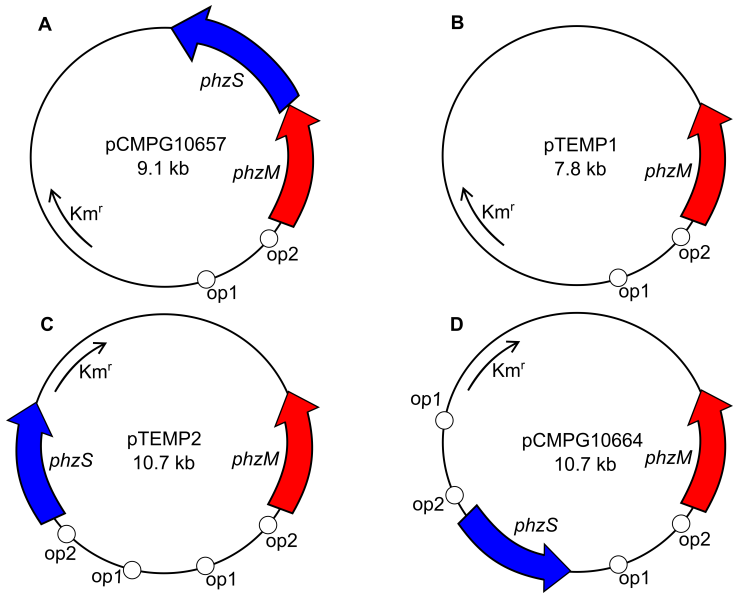


Figure 2.1: Construction of plasmid pCMPG10664

Table 2.1: Overview of plasmids used in this study

Plasmids	Vector	Promoter	Output	Antibiotics
pCMPG10602	pBAD33	P_{BAD}	PCA	25 μg/mL Cm
pCMPG10652	pFPV25	P_{BC}	GFP	100 μg/mL Ap
pCMPG10653	pUCP-MS	P_{BC}	PhzM and PhzS	10 μg/mL Tc
pCMPG10654	pFAJ5301	*3α-hsd* promoter	GFP	150 μg/mL Ery
pCMPG10657	pBBR1MCS-2	*3α-hsd* promoter	PhzM and PhzS	50 μg/mL Km
pCMPG10658	pBAD322K	P_{BAD}	RepA	50 μg/mL Km
pCMPG10659	pUC19	prom. J23100, RBS B0034	RepA	100 μg/mL Ap
pCMPG10660	pUC19	prom. J23114, RBS B0034	RepA	100 μg/mL Ap
pCMPG10661	pUC19	prom. J23104, RBS B0032	RepA	100 μg/mL Ap
pCMPG10662	pUC19	prom. J23110, RBS B0031	RepA	100 μg/mL Ap
pCMPG10663	pUC19	prom. J23110, RBS B0032	RepA	100 μg/mL Ap
pCMPG10664	pBBR1MCS-2	*3α-hsd* promoter	PhzS and PhzM	50 μg/mL Km

Table 2.2: Overview of *E.coli* strains and plasmids used in this study

Strains	**Description**	**Reference**
E. coli MC4100	F$^-$ [araD139]$_{B/r}$ Δ(argF-lac)169* λ^- e14- flhD5301 Δ(fruK-yeiR)725 (fruA25)‡ relA1 rpsL150(strR) rbsR22 Δ(fimB-fimE)632(::IS1) deoC1	[28]
E. coli TOP10	F- mcrA Δ(mrr-hsdRMS-mcrBC) ϕ80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL(Str^R) endA1 λ^-	Invitrogen
Plasmids	**Description**	**Reference**
pCMPG10602	HindIII fragment of pUCP-A2G2 in pBAD33	[208]
pCMPG10652	P$_A$, *hpaA*, P$_{BC}$ of pRA$_2$ cloned EcoRI/XbaI in pFPV25	This work
pCMPG10653	P$_A$, *hpaA*, P$_{BC}$ of pRA$_2$ cloned EcoRI in pUCP-MS	This work
pCMPG10657	operator sites, *phzM*, *phzS* cloned HindIII/EcoRI in pBBR1MCS-2	This work
pCMPG10654	operator sites, P$_{hsd}$ of *C. testosteroni* and *gfp* of pFPV25 cloned EcoRI/XbaI in pFAJ5301	This work
pCMPG10658	*repA* of *C. testosteroni* cloned XbaI/HindIII in pBAD322K	This work
pCMPG10659	promoter J23100, RBS B0034 and *repA* cloned XbaI/HindIII in pUC19	This work
pCMPG10660	promoter J23114, RBS B0034 and *repA* cloned XbaI/HindIII in pUC19	This work
pCMPG10661	promoter J23104, RBS B0032 and *repA* cloned XbaI/HindIII in pUC19	This work
pCMPG10662	promoter J23110, RBS B0031 and *repA* cloned XbaI/HindIII in pUC19	This work
pCMPG10663	promoter J23110, RBS B0032 and *repA* cloned XbaI/HindIII in pUC19	This work
pCMPG10664	operator sites, *phzM*, operator sites, *phzS* cloned BamHI/EcoRI and EcoRI/HindIII in pBBR1MCS-2	This work
pFPV25	promoter trap vector constructed by inserting an EcoRI/HindIII fragment containing a promoterless *gfpmut3* [33] into plasmid pED350 (ColE1, *bla*, *mob*) [37]	[203]
pFPV25.1	promoter rpsM inserted into pFPV25 EcoRI/XbaI	[203]
pUCP-MS	*phzM* and *phzS* of *Pseudomonas aeruginosa* cloned in pUCP-26	[122]
pRA$_2$	P$_A$, *hpaA*, P$_{BC}$, *lacZ* of *E. coli* W ATCC 11105 cloned in pRS551	[155]
pFAJ5301	Cloning vector, pUC18 derivative	[210]
pBAD322K	P$_{BAD}$, *araC* expression vector	[35]
pUC19	Standard cloning vector	[226]
pBBR1MCS-2	Standard cloning vector	[96]

2.2 β-galactosidase assay

β-galactosidase assays were performed in order to verify whether bacteria transformed with pRA_2 could produce a dose-response result (Section 3.3.1).

An overnight culture of bacteria containing pRA_2 was diluted in 1:100 in 200 μL fresh LB medium in the presence of a dilution series of auxins (1 mM, 500 μM, 250 μM and 125 μM) . After an incubation period of 24 hours, 135 μL of culture of each test condition was transferred to a microtiter plate (n=4) whereafter the optical density (OD) of these cultures was measured at 595 nm. To start the assay 10 μL of each culture was transferred eight times to a new microtiter plate, resulting in one column per culture. To start the reaction, 90 μL of ONPG buffer, containing 8 mg/10 mL ortho-nitrophenyl-β-galactoside (ONPG), was added to each well. Immediately after this the cultures were incubated at 37°C and the time of incubation was noted.
The reaction was stopped when a yellow colour appeared by adding 35 μL 1 M Na_2CO_3 and the time of inactivation was noted.
The absorbance of the cultures was measured at 595 nm and 415 nm using Synergy MX microtiter plate reader (Biotek Instruments, Inc.).
The amount of expression of β-galactosidase was expressed in Miller units (U) [128] and was calculated using following formula:

$$1 \text{ Miller unit} = 1000 * \frac{OD_{415} - (1.75 * OD_{595})}{(t * V * OD_{595})} \tag{2.1}$$

OD_{415} = Measured absorbance of the yellow ortho-nitrophenyl
OD_{595}= Measured cell density
t = reaction time in minutes
V = volume of culture assayed in milliliters

2.3 GFP fluorescence assay

To assess the effectiveness of plasmids pCMPG10652 and pCMPG10654 as bioreporters, the GFP production of both plasmids in response to hormones was measured using following protocol (Sections 3.3.2 and 4.3.1).

For pCMPG10652, a 24 hours experiment was performed whereby an overnight culture of bacteria containing pCMPG10652 was diluted 1:100 in 200 μL fresh

Table 2.3: Overview of primers used in this study.

Primers	**Sequence**	**Purpose**
PRO6977	AAAAAAGAATTCAGCAGGCGATCGGTATTG	fwd *hpaA*; EcoRI (pCMPG10652)
PRO7618	AAAAAATCTAGAATCGGTTGTCCGCCTCTAC	rev *hpaA*; XbaI (pCMPG10652)
PRO7995	ATGAATTCATTGAGCAGGCGATCGGTAT	fwd *hpaA*; EcoRI (pCMPG10653)
PRO8160	ATGAATTCGCTGTTTCCTGTGTGATAAAGAA	rev *hpaA*; EcoRI (pCMPG10653)
PRO7047	AAAAAAGAATTCCTCGGCCATGTCAAAGCC	fwd operator sites; EcoRI (pCMPG10654)
S&P0058	CTTCTCCTTTACTCATGTCTTGTCTCCTTT	rev operator sites (pCMPG10654)
S&P0059	AAAGGAGACAAGACATGAGTAAAGGAGAAG	fwd *gfp* (pCMPG10654)
S&P0060	ATTCTAGATGCCTGCAGGAGATTTATTTG	rev *gfp*; XbaI (pCMPG10654)
S&P0061	ATAAGCTTCTCGATCTGAGCTGTGCTGA	fwd *repA*; XbaI (pCMPG10658)
S&P0062	ATTCTAGATACCACGGCGGTCATATGTT	rev repA; HindIII (pCMPG10658)
S&P00137	ATTCTAGACTCGATCTGAGCTGTGCTGA	rev *repA*;XbaI (pCMPG10659-pCMPG10663)
S&P00138	CTAGCTACTAGAGAAATGCACATAGCTTGC	fwd *repA* (pCMPG10660)
S&P00139	ATAAGCTTTTTATGGCTAGCTCAGTCCTAGG	fwd prom., RBS, HindIII (pCMPG10660)
S&P00140	GCAAGCTATGTGCATTTCTCTAGTAGCTAG	rev prom., RBS (pCMPG10660)
S&P00141	CTAGCTACTAGAGAAATGCACATAGCTTGC	fwd *repA* (pCMPG10659)
S&P00142	ATAAGCTTTTGACGGCTAGCTCAGTCCTAGGT	fwd prom., RBS, HindIII (pCMPG10659)
S&P00143	GCAAGCTATGTGCATTTCTCTAGTAGCTAG	rev prom., RBS (pCMPG10659)

Table 2.4: Overview of primers used in this study (Continued).

Primers	**Sequence**	**Purpose**
S&P00144	CAGGAAAGTACTAGATGCACATAGCTTGC	fwd *repA* (pCMPG10661&pCMPG10662)
S&P00145	ATAAGCTTTTGACAGCTAGCTCAGTCCTAGGT	fwd prom., RBS, HindIII (pCMPG10661)
S&P00146	GCAAGCTATGTGCATCTAGTACTTTCCTGT	rev prom., RBS (pCMPG10661&pCMPG10662)
S&P00147	ATAAGCTTTTTACGGCTAGCTCAGTCCTAGGT	fwd prom., RBS, HindIII (pCMPG10662&pCMPG10663)
S&P00148	CAGGAAACCTACTAGATGCACATAGCTTGC	fwd *repA* (pCMPG10663)
S&P00149	GCAAGCTATGTGCATCTAGTAGGTTTCCTG	rev prom., RBS (pCMPG10663)
S&P00421	ATGAATTCCTCGGCCATGTCAAAGCC	fwd operator sitesn; EcoRI (pCMPG10664)
S&P00422	ATTCGAATTATTCATGTCTTGTCTCCTTT	rev operator sites (pCMPG10664)
S&P00423	AAAGGAGACAAGACATGAATAATTCGAAT	fwd *phzM* and *phzS* (pCMPG10664)
S&P00424	ATGGATCCGTGCTGCAAGGCGATTAAGT	rev *phzM* and *phzS*; BamHI (pCMPG10664)
S&P00636	ATGGATCCGTTGAAAGTTCCGATTCAGG	rev *phzM*; BamHI (pCMPG10664)
S&P00637	ATGGATCCCTCGGCCATGTCAAAGCC	fwd operator sites; BamHI (pCMPG10664)
S&P00638	AAAGGAGACAAGACATGAGCGAACCCATC	fwd *phzS* (pCMPG10664)
S&P00639	GATGGGTTCGCTCATGTCTTGTCTCCTTT	rev operator sites (pCMPG10664)
S&P00674	ATAAGCTTCTCGGCCATGTCAAAGCC	fwd operator sites and *phzS*; HindIII (pCMPG10664)
S&P00675	ATGAATTCGCATGCTAGCGTGGC	rev operator sites and *phzS*; EcoRI (pCMPG10664)

LB medium in fluorescence microtiter plates (Greiner Bio-One, 655096). A dilution series of auxins (0.49 μM, 0.98 μM, 1.95 μM, 3.9 μM, 7.81 μM, 15.625 μM, 31.25 μM, 62 μM, 125 μM, 250 μM, 500 μM, 1 mM, 1.5 mM, 2 mM, 3 mM) was added either immediately (induction during lag phase), after three hours (induction during exponential phase) or after 10 hours (induction during stationary phase). Replicates were grown in parallel on the same day. The plates were cultured for 24 h at 37°C during which the absorbance at 595 nm and the fluorescence intensity (excitation 488 nm, emission 511 nm) were measured by the Synergy MX microtiter plate reader (Biotek Instruments, Inc.) every 2 hours. The measured OD_{595} values were used to correct the fluorescence intensity for bacterial growth.
For further experiments, an overnight culture of bacteria containing pCMPG10652 was diluted 1:100 in 200 μL fresh LB medium in fluorescence microtiter plates (Greiner Bio-One, 655096). A dilution series of auxins (0.49 μM, 0.98 μM, 1.95 μM, 3.9 μM, 7.81 μM, 15.625 μM, 31.25 μM, 62 μM, 125 μM, 250 μM, 500 μM, 1 mM, 1.5 mM, 2 mM, 3 mM) was added after three hours (during exponential phase) for optimal results. Replicates were grown in parallel on the same day. The plates were cultured for 4-8 h at 37°C after which the absorbance at 595 nm and the fluorescence intensity (excitation 488 nm, emission 511 nm) were measured by the Synergy MX microtiter plate reader. The measured OD_{595} values were used to correct the fluorescence intensity for bacterial growth.
For experiments with bacteria containing pCMPG10654 in combination with one of the following plasmids, pCMPG10658-pCMPG10663, an overnight culture was diluted 1:100 in 200 μL fresh LB medium in fluorescence microtiter plates (Greiner Bio-One, 655096). A dilution series of steroid hormones (0.49 μM, 0.98 μM, 1.95 μM, 3.9 μM, 7.81 μM, 15.625 μM, 31.25 μM, 62 μM, 125 μM, 250 μM, 500 μM, 1 mM, 1.5 mM, 2 mM, 3 mM) was added. Replicates were grown in parallel on the same day. The plates were cultured for 24 h at 37°C after which the absorbance at 595 nm and the fluorescence intensity (excitation 488 nm, emission 511 nm) were measured by the Synergy MX microtiter plate reader. The measured OD_{595} values were used to correct the fluorescence intensity for bacterial growth.

2.4 Fluorescence correction factor

To address the effects of steroid hormones on both fluorescence intensity and absorbance, a correction factor was determined and applied to the measured results (Section 4.3.1). The factor was determined by measuring the fluorescence intensity of bacteria, transformed with plasmid pFPV25.1, in the presence of a dilution series of testosterone from 250 μM to 3 mM. Plasmid pFPV25.1 contains the *gfp* gene preceded by constitutive promoter rpsM. The fluorescence

intensity and OD_{595} were measured immediately after the addition of the dilution series of testosterone. The correction factor for the fluorescence intensity was determined for each concentration of testosterone by dividing the measured fluorescence intensity produced in the absence of testosterone with the measured fluorescence intensity of each concentration of testosterone. The correction factor for the OD_{595} was determined for each concentration by subtracting the measured OD_{595} of each concentration with the measured OD_{595} in the absence of testosterone because the OD_{595} increases linearly in the presence of testosterone. This gives the following correction factor for each result:

$$\frac{(F_m * CF_F) - F_{m0}}{(OD_{595m} - CF_{OD_{595}}) - OD_{595m0}} \tag{2.2}$$

F_m = Measured fluorescence intensity
CF_F = Correction factor fluorescence intensity
F_{m0} = Measured fluorescence intensity in absence of steroid hormones
OD_{595m} = Measured OD_{595}
$CF_{OD_{595}}$= Correction factor OD_{595}
OD_{595m0} = Measured OD_{595} in absence of steroid hormones

Table 2.5: Correction factor for fluorescence intensity and OD_{595} for each concentration of steroid hormone

Concentration	$\mathbf{CF}_F$	$\mathbf{CF}_{OD_{595}}$
3 mM	1.952	0.607
2 mM	1.773	0.437
1.5 mM	1.421	0.282
1 mM	1.219	0.144
500 μM	1.062	0.036
250 μM	1.004	0.008

2.5 Pyocyanin assay

To assess the effectiveness of plasmid pCMPG10653 as bioreporter, its pyocyanin production in response to auxins was measured using following protocol (Sections 3.3.3).

An overnight culture of bacteria containing both pCMPG10602 and pCMPG10653 was diluted 1:100 in 5 mL fresh LB medium in tubes in the presence of a dilution

series of auxins (0.49 μM, 0.98 μM, 1.95 μM, 3.9 μM, 7.81 μM, 15.625 μM, 31.25 μM, 62 μM, 125 μM, 250 μM, 500 μM, 1 mM, 1.5 mM, 2 mM, 3 mM). The tubes were incubated for 24h at 37°C after which a Cyclic Voltammetry (CV) experiment was performed. In a CV measurement the potential of the working electrode is increasing linearly over time. When a certain set potential was reached the potential is similarly decreased linearly. During one experiment this can happen multiple times. The current at the working electrode is measured and plotted in function of the applied potential, this results in a cyclic voltammogram [92].
The CV measurements were performed by using a μAutolab type III computer-controlled potentiostat (Metrohm) on a 60EcoMEA-Glass (Multi Channel Systems). The experimental setup was a three-electrode configuration with a multielectrode array (MEA) as the working electrode, a Red Rod reference electrode (Radiometer Analytical) and a coiled platinum wire as counter electrode.
The MEA is connected to the potentiostat through a printed circuit board which allows to connect the MEA contact pads with the connection clamps of the potentiostat.
During the experiment the potentiostat cycled 20 times from -0.5 V until 0.1 V, and back again, with a 2 mV step potential.

2.6 Fluorescence and pyocyanin assay data analysis

The analysis of the data obtained with both the fluorescence assay and the pyocyanin assay is done using Graphpad Prism 5. To start the analysis, the binary logarithm (log_2) of the induction hormone concentrations was taken. Afterwards the data was fitted using a non-linear regression with a log(agonist) vs response – variable slope (four parameters) model. The equation used for this fitting is:

$$Y = \frac{Bottom + (Top - Bottom)}{1 + 10^{Log(EC_{50}) - X} * HillSlope} \tag{2.3}$$

Bottom = Lower plateau of fluorescence intensity (a.u.) / induced current
Top = Upper plateau of fluorescence intensity (a.u.) / induced current
EC_{50} = Concentration of hormone that gives a response halfway between Top and Bottom
HillSlope = Measure for the steepness of the slope

Top and Bottom values were used to determine the upper and lower detection limits of the bioreporter. The R^2-values, a measure for how well the generated curve fits the data, were used to determine the dose-responseness of the response.

Chapter 3

Design and construction of auxin bioreporters

3.1 Abstract

Auxins are hormones that play an important role in the growth and development of plants. To be able to study the effects of endogenous and exogenous auxins on plant growth, sensitive detection methods are needed. In this chapter, the design and construction of two different whole-cell bacterial auxin bioreporters is illustrated. Both use the auxin responsive protein HpaA as an input module. The first auxin bioreporter incorporates the *gfp* gene to produce a fluorescent bioreporter. The second bioreporter utilizes the genes *phzM* and *phzS* to produce a pyocyanin-producing bioreporter whose product can be measured electrochemically in an integrated biosensor.
The fluorescent bioreporter can detect auxins 4-hydroxyphenylacetic acid (4-HPA) and 2-phenylacetic acid (PAA) concentrations from 31.25 μM to 3 mM in a dose-response manner.
The pyocyanin-producing biosensor is able to detect 4-HPA concentrations from 1.95 μM to 15.63 μM and PAA concentrations from 15.63 μM to 125 μM.

3.2 Introduction

The auxin reporters are whole-cell bacterial bioreporters that contain an input module and two different output modules. The input module consists of an auxin responsive protein, transcription factor HpaA, which detects the presence of auxins. After binding the auxin, HpaA transduces the signal by binding to promoter P_{BC} and subsequently activates the expression of the output module, GFP or pyocyanin.
In this chapter, the design and construction of two whole-cell bacterial auxin bioreporters is described. Section 3.2.1 reports on a suitable auxin responsive protein, HpaA, that will be used as an input module for the bioreporter. Section 3.3.2 describes the combination of HpaA with a GFP-producing output module, resulting in a fluorescence producing auxin bioreporter. Next, section 3.3.3 describes the combination of the responsive protein with a pyocyanin-producing output module. This pyocyanin-producing bioreporter was integrated with a MEA, resulting in an auxin biosensor that produces redox-active molecule pyocyanin in the presence of auxins. This chapter ends with a discussion of the results of both bioreporters in section 3.4.

3.2.1 Description of auxin responsive protein HpaA

A functional whole-cell auxin bioreporter requires an auxin responsive protein that functions as an input module. This input module is a protein or other biological component that can bind to auxins and signal their presence in the cell. Such a responsive protein can often be found in catabolic pathways because these pathways are all adapted to the metabolism of specific compounds and should only be active in the presence of these compounds. Catabolic pathways regularly possess transcriptional regulators that regulate the expression of metabolizing enzymes by binding the compound that is to be metabolized [205]. The ability of a regulator to induce the expression of certain genes after binding a specific compound allows it to be used as a responsive protein for the detection of that specific compound.
Prieto *et al.* have identified several operons involved in the catabolic pathways of auxins 2-phenylacetic acid (PAA) (Fig. 1.6–B (page 17)) and its derivative 4-hydroxyphenylacetic acid (4-HPA) (Fig. 1.6–F (page 17)) in *E. coli* W (ATCC 11105) [56, 59], including operon *hpaBC* which provided the required auxin responsive protein [155]. Operon *hpaBC* is present in *E. coli* W (ATCC 11105), a bacterial strain that has the innate ability to metabolize aromatic compounds like 4-HPA, contrary to *E. coli* K-12 strains which do not possess this ability [154].

Regulation of HpaBC

hpaBC codes for a two component aromatic hydroxylase, HpaBC, responsible for the first step of the 4-HPA catabolism. Its expression is regulated by transcription factor HpaA, encoded by gene *hpaA*, which is constitutively expressed by promoter P_A.
Once HpaA is bound to 4-HPA a conformation change occurs, allowing the HpaA–4-HPA complex to bind to promoter P_{BC}. This induces the expression of HpaBC, downstream of P_{BC}, which in turn will break down 4-HPA (Fig. 3.1) [154, 155].
The ability of HpaA to induce expression of genes downstream of promoter P_{BC} after binding 4-HPA, allows it to be used as biological responsive protein for a 4-HPA bioreporter. PAA has a similar structure to 4-HPA and can also bind to HpaA [155], which can therefore be used to detect both 4-HPA and PAA. HpaA was also used in combination with indole-3-acetic acid (IAA) (Fig. 1.6–A (page 17)), another auxin with a different structure from 4-HPA and PAA, to verify the selectivity of the bioreporter.

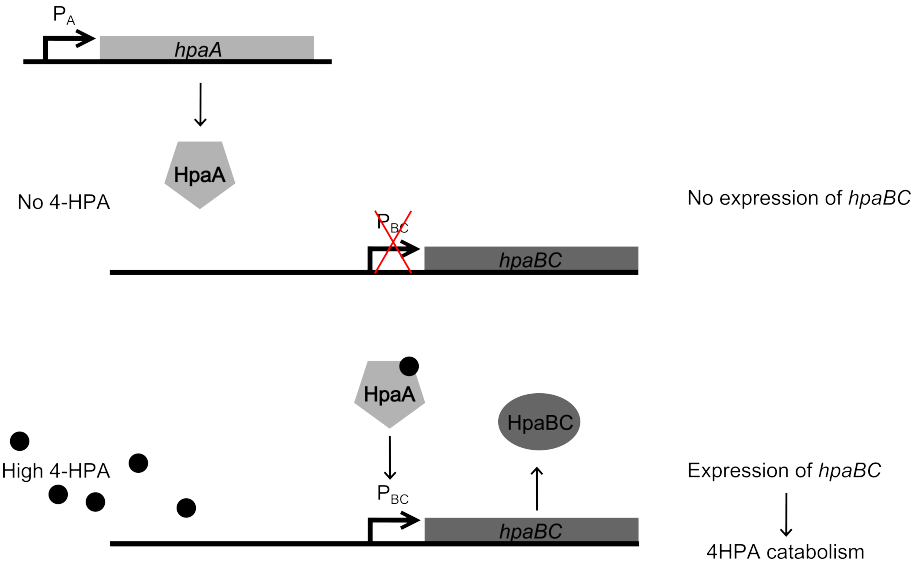


Figure 3.1: Regulation of operon *hpaBC* by HpaA, encoded by gene *hpaA*. (top) HpaA does not bind to promoter P_{BC} in the absence of 4-HPA, therefore HpaBC is not expressed. (bottom) When 4-HPA binds to HpaA, HpaA in turn binds to P_{BC} and promotes the expression of *hpaBC*.

Prieto *et al.* constructed pRA_2, a plasmid containing constitutive promoter P_A, gene *hpaA*, auxin responsive promoter P_{BC} and the *lacZ* gene (Fig. 3.4–A (page 52)), to study the regulation of *hpaBC* [155]. *E. coli* MC4100, a K-12 derived strain which cannot break down 4-HPA, was transformed with pRA_2. If 4-HPA is present in the medium, these bacteria will produce β-galactosidase, which is measurable by adding a chromogenic substrate such as CPRG. Plasmid pRA_2 was used as a starting point for the construction of both the fluorescent and the electrochemical whole-cell bacterial auxin bioreporter.

3.3 Results

3.3.1 The activity of HpaA displays a dose-response relationship

The results obtained with an ideal bioreporter describe a dose-response relationship between the bioreporter and the analyte that is to be measured. The expression of the reporter molecules should hereby be equivalent to the amount of analyte present. pRA_2/MC4100 was procured from the Department of Molecular Microbiology, Centro de Investigaciones Biológicas, Consejo Superior de Investigaciones Científicas in Madrid, Spain, to verify whether it can display such a dose-equivalent response in response to auxins.

pRA_2/MC4100 displays a dose-equivalent response

The production of β-galactosidase by pRA_2/MC4100 bacteria in response to 4-HPA was measured to determine whether 4-HPA can induce a dose-equivalent response. Figure 3.2 shows the outcome of the expression test of strain pRA_2/MC4100 after an incubation of 24 hours in the presence of 4-HPA concentrations of either 0 M, 125 μM, 250 μM, 500 μM or 1 mM by showing the amount of β-galactosidase produced in function of the binary logarithm ($\log_2$) of the auxin concentration. As can be seen in the figure, bacteria grown in the presence of higher concentrations of 4-HPA induce production of a higher amount of β-galactosidase. The linear part of the sigmoidal fit of the data is situated between binary logarithmic values -5–0 and shows that a dose-equivalent production of β-galactosidase can be achieved in response to 4-HPA concentrations between 31.25 μM and 1 mM. However, more data points are needed for a more accurate determination of the upper and lower detection limits. Since pRA_2/MC4100 can detect 4-HPA in a dose-response manner, responsive

protein HpaA was used for further development of the auxin bioreporters.

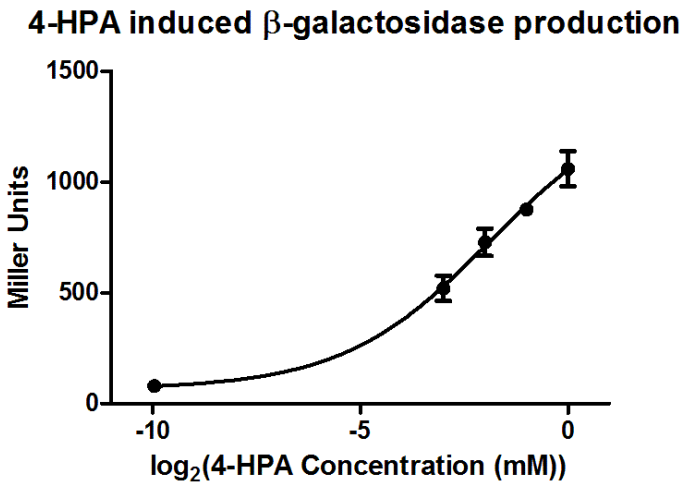


Figure 3.2: β-galactosidase production of strain pRA$_2$/MC4100 in function of the logarithm of 4-HPA (concentrations 0 M, 125 μM, 250 μM, 500 μM, 1 mM). β-galactosidase production is measured after 24 h of incubation at 37°C. Error bars show standard deviation, based on three replicates. The data was fitted using a sigmoidal dose-response fit ($R^2 = 0.9767$).

pRA$_2$/Top10 displays a dose-equivalent response

The β-galactosidase expression of pRA$_2$/TOP10 in response to 4-HPA, PAA and IAA was measured and compared to that of pRA$_2$/MC4100 because *E. coli* TOP10 is a model *E. coli* strain often used as recipient cell in transformations. Figure 3.3–A compares the β-galactosidase expression between both strains of *E. coli* in the presence of 4-HPA concentrations of either 0 M, 125 μM, 250 μM, 500 μM or 1 mM. pRA$_2$/TOP10 and pRA$_2$/MC4100 both display a dose-equivalent response in response to 4-HPA, yet pRA$_2$/MC4100 produces higher amounts of β-galactosidase (Fig. 3.3–A). pRA$_2$/TOP10 appears to not have reached a plateau yet, but more data points are needed for a more accurate determination of the upper and lower detection limits.

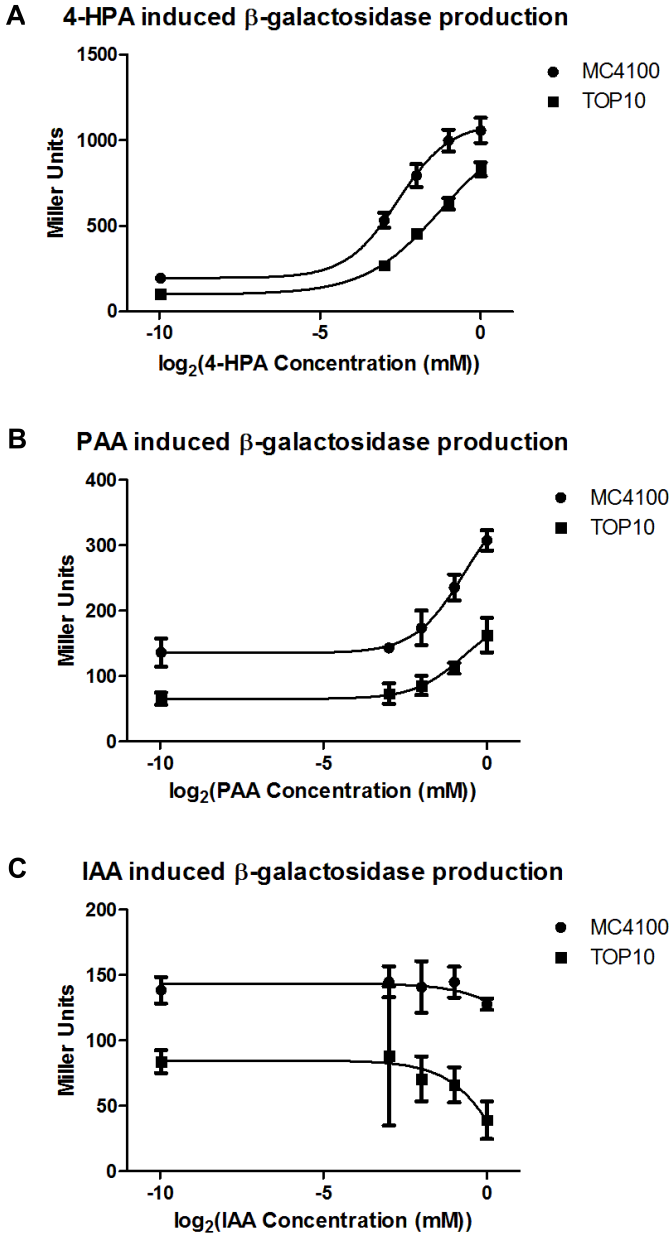


Figure 3.3: β-galactosidase production of strains pRA_2/MC4100 and pRA_2/TOP10 in response to A. 4-HPA B. PAA C. IAA (concentrations 0 M, 125 μM, 250 μM, 500 μM, 1 mM). β-galactosidase production is measured after 24 h of incubation at 37°C. Error bars show standard deviation, based on three replicates. The data was fitted using a sigmoidal dose-response fit.

A dose-equivalent response can be seen for PAA in both strains (Fig. 3.3–B) as well. However, the linear part of the sigmoidal fit only starts around 125 μM (binary logarithmic value: -3) and a plateau has not yet been reached. Additionally, the amount of produced β-galactosidase in response to PAA is four times less as compared to β-galactosidase production in the presence of 4-HPA.
Neither pRA_2/MC4100 nor pRA_2/TOP10 produce more β-galactosidase in response to IAA, than in its absence (Fig. 3.3–C).
The average expression of β-galactosidase in the presence of auxins is slightly lower in pRA_2/TOP10 bacteria, but in absence of either 4-HPA or PAA, there is less β-galactosidase activity in TOP10 than in MC4100 bacteria. There is therefore less leaky expression of β-galactosidase in TOP10 than in MC4100 bacteria. And while pRA_2/TOP10 bacteria produce lower amounts of β-galactosidase, they have a better signal-to-noise ratio than pRA_2/MC4100 bacteria. These results show that *E. coli* TOP10 is suitable as a genetic background for the HpaA input module and that the auxin responsive protein is selective for 4-HPA and PAA.

3.3.2 GFP as reporter system

Design of a GFP-producing auxin bioreporter

The LacZ reporter system is a labour-intensive and time-consuming output module due to a lengthy preparation process. To improve the usability of the bioreporter, the LacZ reporter system was replaced by the GFP reporter system, resulting in a bioreporter with a fluorescence output module.
The GFP reporter system consists only of gene *gfp* derived of plasmid pFPV25. pFPV25 is a plasmid often used to study the activity of unknown promoters [21] because it contains the *gfp* gene preceded by a multiple cloning site (MCS) (Fig. 3.4–B) [203].
The DNA sequence of pRA_2 containing the constitutive P_A promoter, the *hpaA* gene followed by 4-HPA responsive promoter P_{BC} was amplified and placed upstream of the *gfp* gene in plasmid pFPV25. This resulted in plasmid pCMPG10652 (Fig. 3.4–C), as described in materials and methods (chapter 2).

4-HPA and PAA induce a dose-equivalent production of GFP

A series of expression tests was performed to determine the validity of pCMPG10652/TOP10 as a bioreporter. First and foremost pCMPG10652/TOP10 should produce a dose-equivalent response of GFP production in the presence

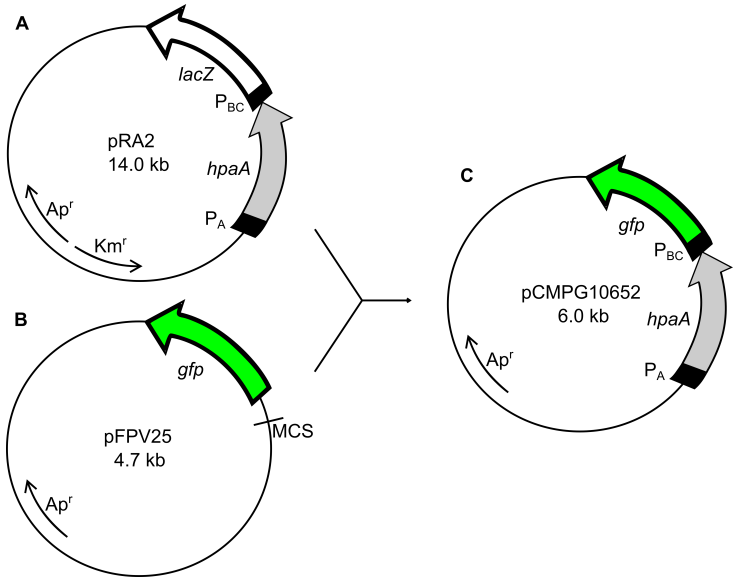


Figure 3.4: Construction of plasmid pCMPG10652. Constitutive P_A promoter, the *hpaA* gene and 4-HPA responsive promoter P_{BC} were copied from plasmid pRA_2 and placed upstream of the *gfp* gene in plasmid pFPV25. A. pRA_2 B. pFPV25 C. pCMPG10652

of auxins. Secondly, the ideal time point of induction, during the growth of the bioreporter culture, was to be determined. Cultures of bacteria display four distinct phases during growth. During lag phase (0–2 h), bacteria adapt to growth conditions, the exponential phase (2–6 h) is characterized by cell doubling. After depletion of essential nutrients the growth rate and the death rate are equal, this phase is called the stationary phase (6–10 h) as a plateau in population level has been reached. And finally the bacteria die during the death phase (+10 h). Assays were performed to verify which phase of growth was the best time point of induction for pCMPG10652/TOP10 bacteria by inducing the bacteria during either lag phase, exponential phase or stationary phase. Thirdly, all induced pCMPG10652/TOP10 bacteria were measured every 2 hours for 24 hours to determine the length of time needed to produce a dose-equivalent response. All experiments were performed in the presence of either 4-HPA, PAA or IAA.

The results of the 24 hours during experiments of pCMPG10652/TOP10 bacteria induced by either 4-HPA, PAA or IAA, during either lag phase, exponential phase or stationary phase are shown in figures 3.5–3.13 where the amount of

fluorescence produced is shown in function of the binary logarithm of the auxin concentration. All data is fitted using a sigmoidal dose-response fit as described in materials and methods, section 2.6. The R^2 -values, obtained from these fits, are a measure for how well the generated curve fits the data and will be used as an objective standard, they are listed in table 3.1.
The figures and their respective R^2-values show that pCMPG10652/TOP10 bacteria achieve a dose-response in the presence of 4-HPA and PAA. They also show that for both 4-HPA and PAA, the best dose-response results are obtained after induction during the exponential phase of growth since the average of the R^2-values of the fits is highest for the exponential phase for both auxins (average R^2-values for 4-HPA are 0.9843, 0.8968 and 0.8905 for exponential, lag and stationary phase respectively, for PAA they are 0.9407, 0.9366 and 0.8717). IAA elicits no production of GFP, independent of induction during a particular phase, as can be seen in figures 3.11–3.13 (despite high R^2-values of the fits of the data of the initial time points).

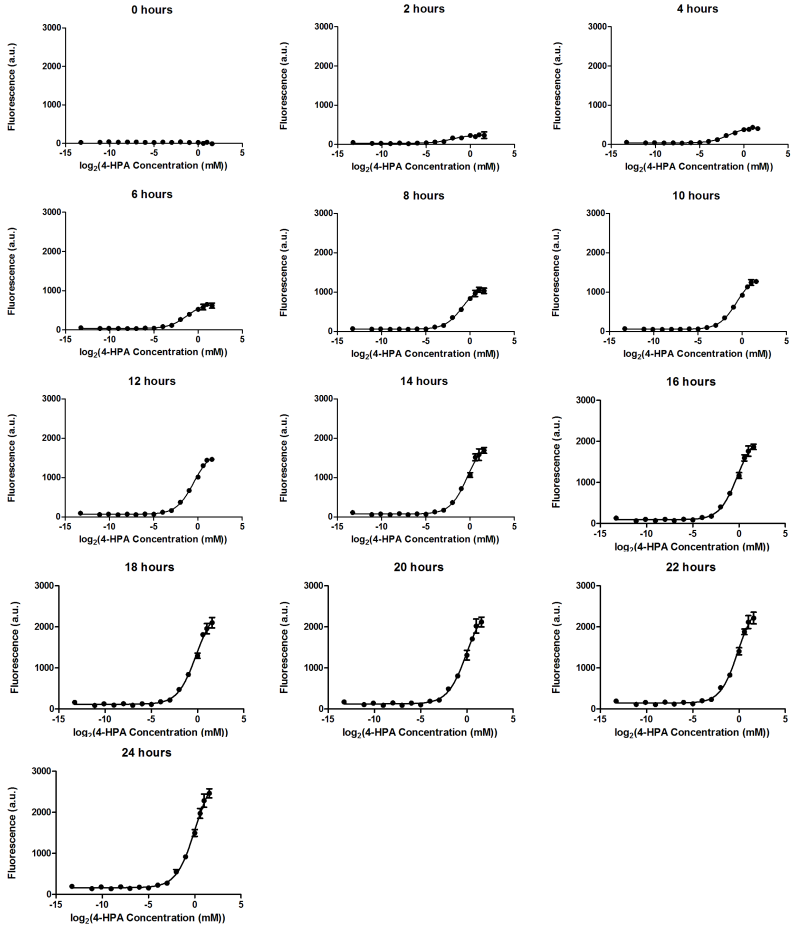


Figure 3.5: GFP production of strain pCMPG10652/TOP10 in response to 4-HPA, induced during exponential phase (concentrations 0 M, 0.49 μM, 0.98 μM, 1.95 μM, 3.91 μM, 7.81, μM, 15.63 μM, 31.25 μM, 62.50 μM, 125 μM, 250 μM, 500 μM, 1 mM, 1.5 mM, 2 mM, 3 mM) measured during 24 hours, with 2 hour steps. Error bars show standard deviation, based on three replicates. The data was fitted using a sigmoidal dose-response fit.

Table 3.1: List of R^2-values obtained from the sigmoidal dose-response fits of the GFP production in response to auxin 4-HPA, PAA or IAA. The R^2-value of each time point (0–24 hours) is shown for all three moments of induction (exponential, lag or stationary phase). The average of the R^2-values over all time points per moment of induction is shown in the last row. Empty boxes denote data for which no fit could be obtained.

	4-HPA			**PAA**			**IAA**		
Δh	exp	lag	stat	exp	lag	stat	exp	lag	stat
0		0.2725	0.1445			0.1421		0.7459	0.4842
2	0.9148	0.5490	0.9049	0.7054		0.7830	0.5004	0.4935	0.3455
4	0.9777	0.9472	0.9426	0.8753	0.8212	0.8976	0.5152	0.677	0.3778
6	0.9853	0.9799	0.9407	0.9344	0.8680	0.9199	0.4228	0.6707	0.4683
8	0.9915	0.9896	0.9497	0.9582	0.9045	0.9254	0.5316	0.5715	0.3938
10	0.9962	0.9885	0.9591	0.9764	0.9298	0.9427	0.3933	0.4202	0.3556
12	0.9956	0.9932	0.9664	0.9763	0.9530	0.9728		0.4936	0.2207
14	0.9902	0.9908	0.9613	0.9800	0.9589	0.9573		0.4406	
16	0.9931	0.9868	0.9631	0.9798	0.9718	0.9602		0.3465	0.1537
18	0.9928	0.9892	0.9560	0.9745	0.9764	0.9670		0.1724	
20	0.9905	0.9904	0.9576	0.9772	0.9758	0.9534	0.1003	0.3165	0.1804
22	0.9912	0.9920	0.9643	0.9809	0.9710	0.9534	0.1108	0.3660	
24	0.9934	0.9896	0.9665	0.9699	0.9724	0.9567			0.1160
Average	0.9843	0.8968	0.8905	0.9407	0.9366	0.8717	0.3678	0.4762	0.3096

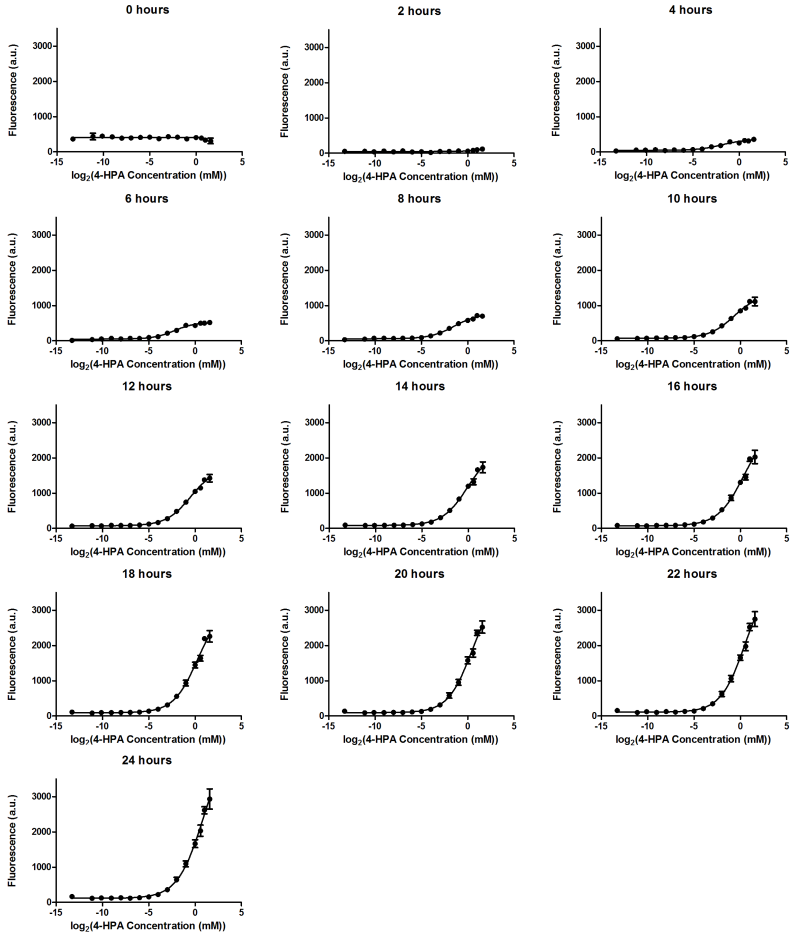


Figure 3.6: GFP production of strain pCMPG10652/TOP10 in response to 4-HPA, induced during lag phase (concentrations 0 M, 0.49 μM, 0.98 μM, 1.95 μM, 3.91 μM, 7.81, μM, 15.63 μM, 31.25 μM, 62.50 μM, 125 μM, 250 μM, 500 μM, 1 mM, 1.5 mM, 2 mM, 3 mM) measured during 24 hours, with 2 hour steps. Error bars show standard deviation, based on three replicates. The data was fitted using a sigmoidal dose-response fit.

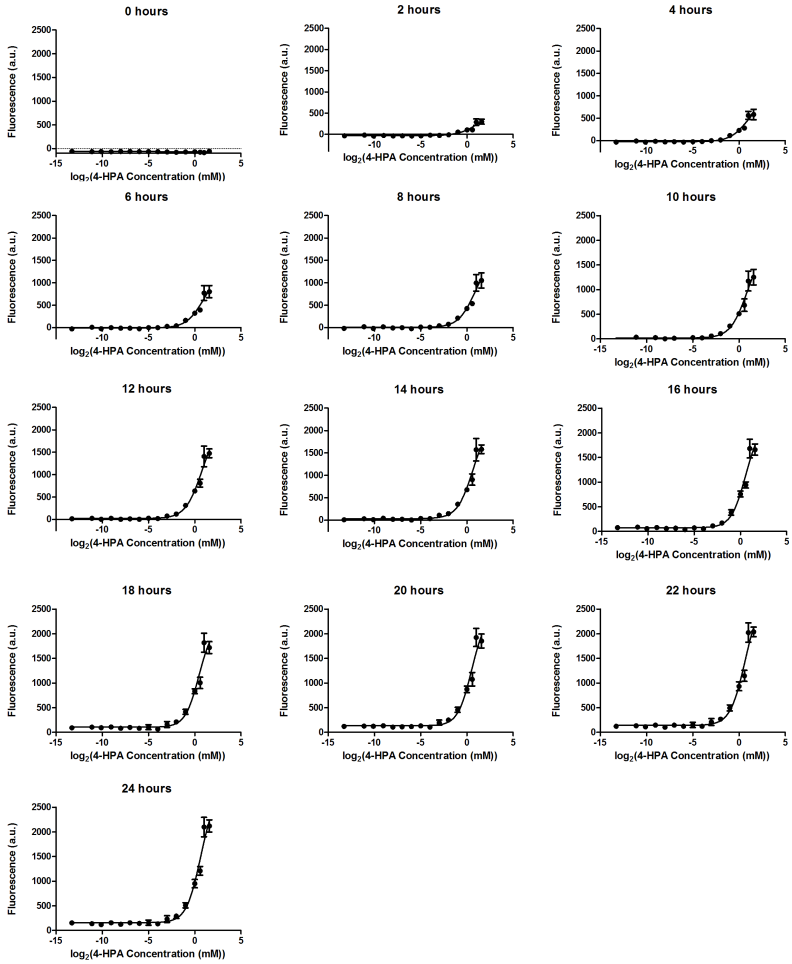


Figure 3.7: GFP production of strain pCMPG10652/TOP10 in response to 4-HPA, induced during stationary phase (concentrations 0 M, 0.49 μM, 0.98 μM, 1.95 μM, 3.91 μM, 7.81, μM, 15.63 μM, 31.25 μM, 62.50 μM, 125 μM, 250 μM, 500 μM, 1 mM, 1.5 mM, 2 mM, 3 mM) measured during 24 hours, with 2 hour steps. Error bars show standard deviation, based on three replicates. The data was fitted using a sigmoidal dose-response fit.

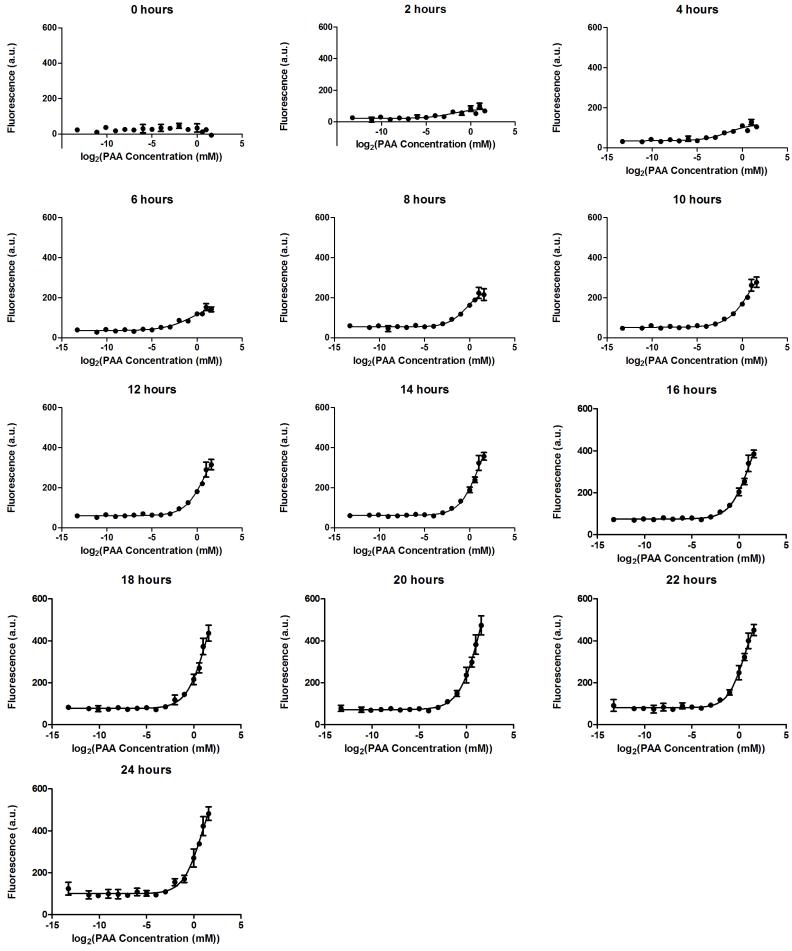


Figure 3.8: GFP production of strain pCMPG10652/TOP10 in response to PAA, induced during exponential phase (concentrations 0 M, 0.49 μM, 0.98 μM, 1.95 μM, 3.91 μM, 7.81, μM, 15.63 μM, 31.25 μM, 62.50 μM, 125 μM, 250 μM, 500 μM, 1 mM, 1.5 mM, 2 mM, 3 mM) measured during 24 hours, with 2 hour steps. Error bars show standard deviation, based on three replicates. The data was fitted using a sigmoidal dose-response fit.

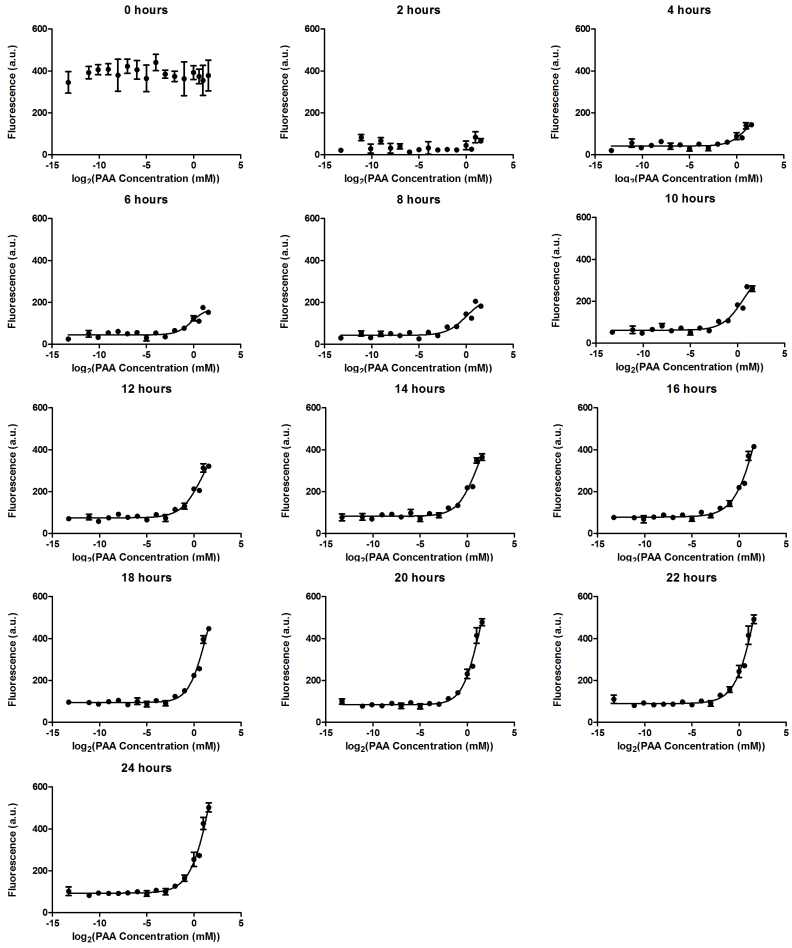


Figure 3.9: GFP production of strain pCMPG10652/TOP10 in response to PAA, induced during lag phase (concentrations 0 M, 0.49 μM, 0.98 μM, 1.95 μM, 3.91 μM, 7.81, μM, 15.63 μM, 31.25 μM, 62.50 μM, 125 μM, 250 μM, 500 μM, 1 mM, 1.5 mM, 2 mM, 3 mM) measured during 24 hours, with 2 hour steps. Error bars show standard deviation, based on three replicates. The data was fitted using a sigmoidal dose-response fit.

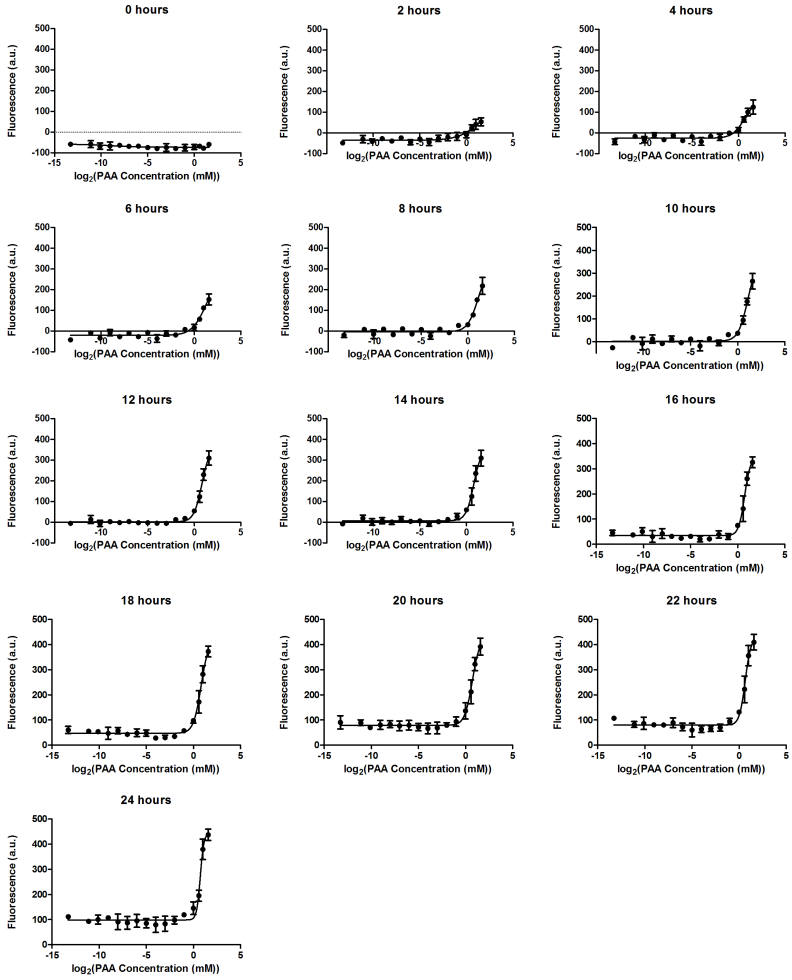


Figure 3.10: GFP production of strain pCMPG10652/TOP10 in response to PAA, induced during stationary phase (concentrations 0 M, 0.49 μM, 0.98 μM, 1.95 μM, 3.91 μM, 7.81, μM, 15.63 μM, 31.25 μM, 62.50 μM, 125 μM, 250 μM, 500 μM, 1 mM, 1.5 mM, 2 mM, 3 mM) measured during 24 hours, with 2 hour steps. Error bars show standard deviation, based on three replicates. The data was fitted using a sigmoidal dose-response fit.

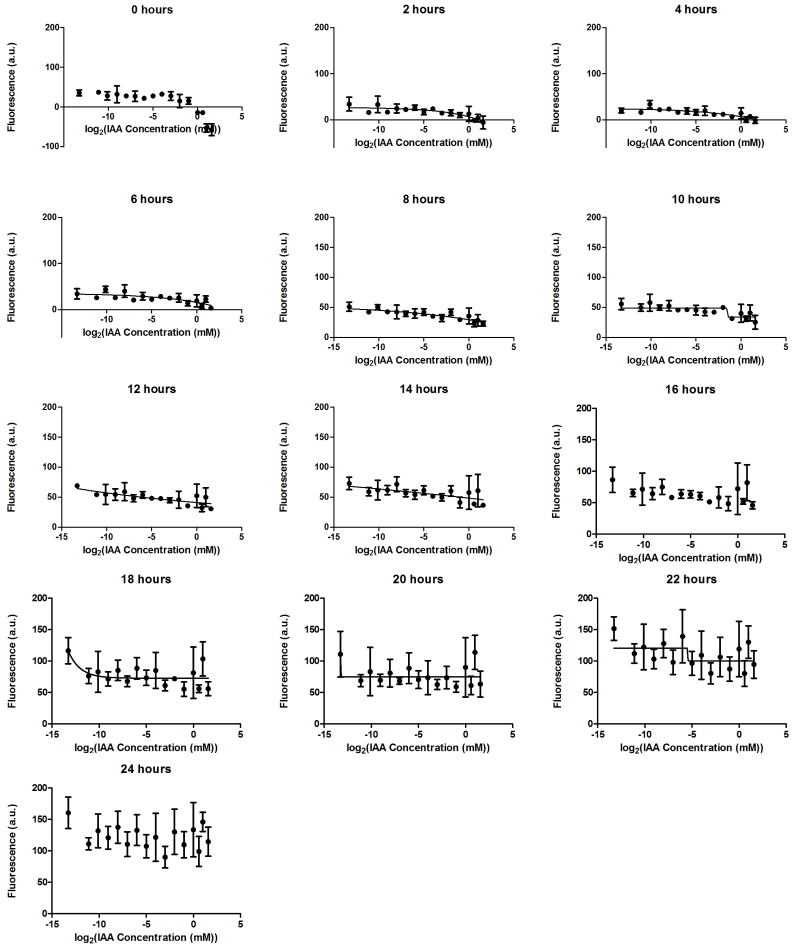


Figure 3.11: GFP production of strain pCMPG10652/TOP10 in response to IAA, induced during exponential phase (concentrations 0 M, 0.49 μM, 0.98 μM, 1.95 μM, 3.91 μM, 7.81, μM, 15.63 μM, 31.25 μM, 62.50 μM, 125 μM, 250 μM, 500 μM, 1 mM, 1.5 mM, 2 mM, 3 mM) measured during 24 hours, with 2 hour steps. Error bars show standard deviation, based on three replicates. The data was fitted using a sigmoidal dose-response fit.

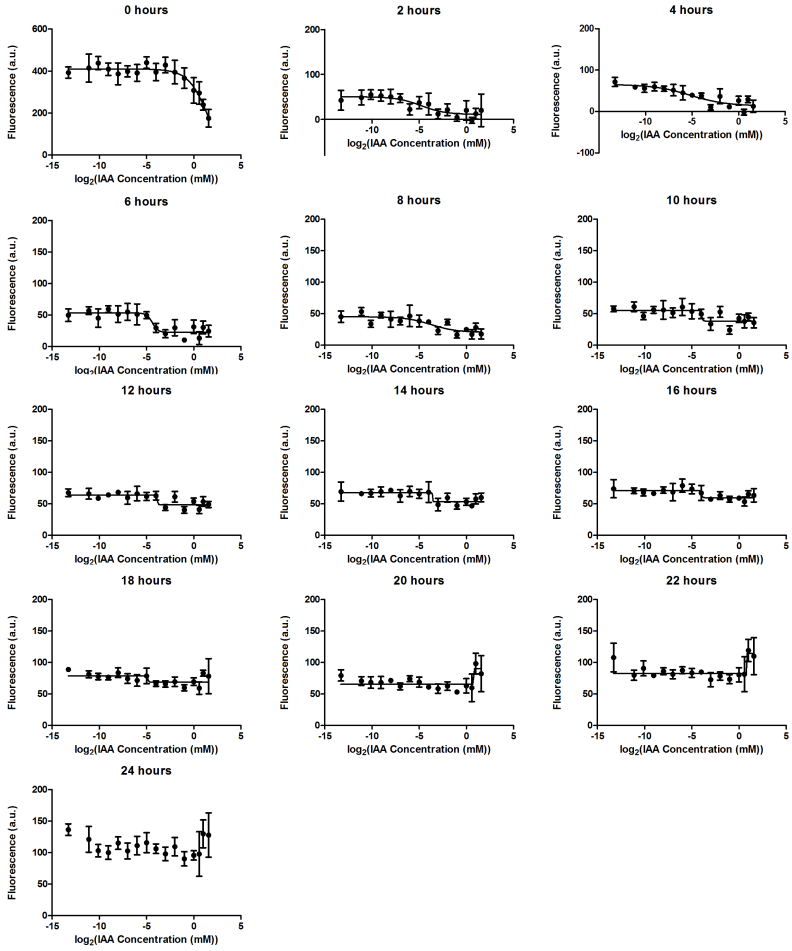


Figure 3.12: GFP production of strain pCMPG10652/TOP10 in response to IAA, induced during lag phase (concentrations 0 M, 0.49 μM, 0.98 μM, 1.95 μM, 3.91 μM, 7.81, μM, 15.63 μM, 31.25 μM, 62.50 μM, 125 μM, 250 μM, 500 μM, 1 mM, 1.5 mM, 2 mM, 3 mM) measured during 24 hours, with 2 hour steps. Error bars show standard deviation, based on three replicates. The data was fitted using a sigmoidal dose-response fit.

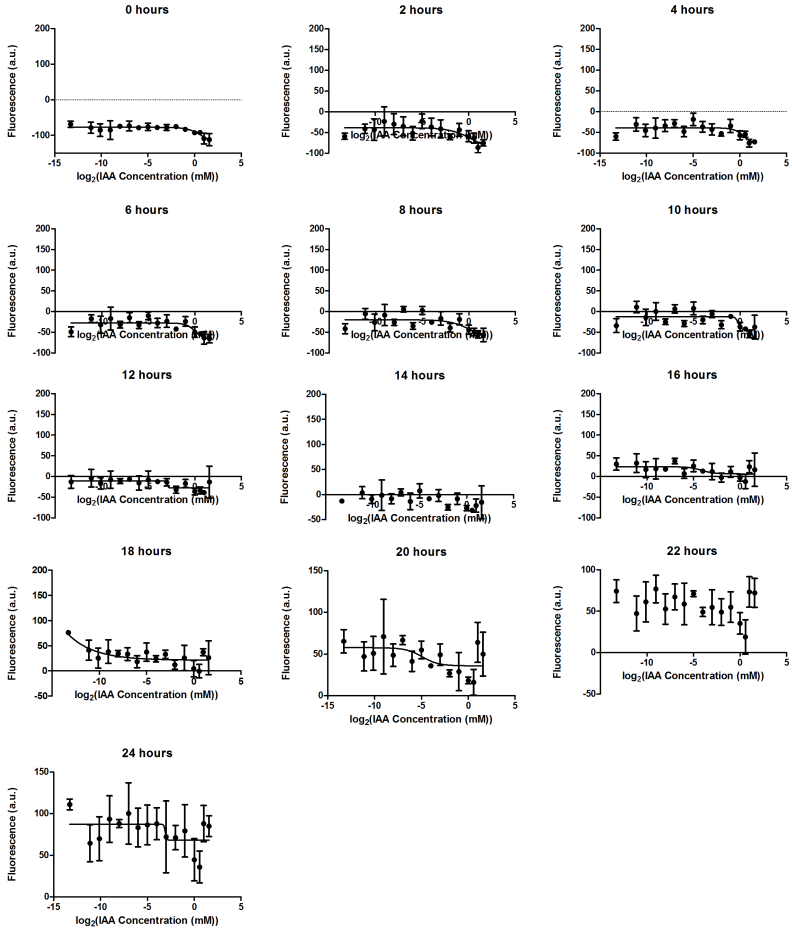


Figure 3.13: GFP production of strain pCMPG10652/TOP10 in response to IAA, induced during stationary phase (concentrations 0 M, 0.49 μM, 0.98 μM, 1.95 μM, 3.91 μM, 7.81, μM, 15.63 μM, 31.25 μM, 62.50 μM, 125 μM, 250 μM, 500 μM, 1 mM, 1.5 mM, 2 mM, 3 mM) measured during 24 hours, with 2 hour steps. Error bars show standard deviation, based on three replicates. The data was fitted using a sigmoidal dose-response fit.

The R^2-values were used as an objective standard to determine the length of time, after induction, needed for pCMPG10652/TOP10 bacteria to produce a dose-equivalent response. The R^2-values of the fits of the data obtained after 2–4 hours induction in the presence of 4-HPA, induced during exponential phase, are higher than 0.9100, which suggests that a dose-response result can be achieved after 2–4 hours of incubation. Figure 3.14–A shows the GFP production of pCMPG10652/TOP10 bacteria after 2–4 hours in the presence of 4-HPA, induced during exponential phase. A dose-response result can clearly be seen, even though the amount of fluorescence produced after 2–4 hours of incubation is markedly lower than after 24 hours of incubation.

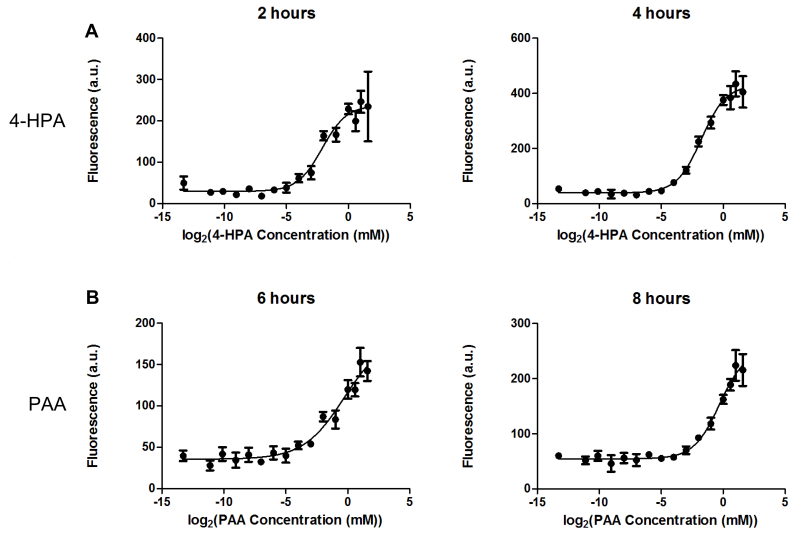


Figure 3.14: GFP production of strain pCMPG10652/TOP10 after 2-4 hours in response to A. 4-HPA B. PAA (concentrations 0 M, 0.49 μM, 0.98 μM, 1.95 μM, 3.91 μM, 7.81, μM, 15.63 μM, 31.25 μM, 62.50 μM, 125 μM, 250 μM, 500 μM, 1 mM, 1.5 mM, 2 mM, 3 mM), induced during exponential phase. Error bars show standard deviation, based on three replicates. The data was fitted using a sigmoidal dose-response fit.

In figure 3.14–B a closer look at the GFP production of pCMPG10652/TOP10 bacteria after 6–8 hours in the presence of PAA is shown, also induced during exponential phase. Here too a dose-response result can be seen, while similarly less fluorescence is produced than after 24 hours of incubation. The R^2-values of the fits shown in figure 3.14–B are higher than 0.9300, corroborating the

dose-response result pCMPG10652/TOP10 bacteria produce in the presence of PAA. Similar to the results obtained with the *lacZ* reporter system, PAA elicits less GFP production than 4-HPA
For both 4-HPA and PAA the linear part of the sigmoidal fit starts at 31.25 μM (binary logarithmic value: -5) and a plateau is reached at 3 mM (binary logarithmic value: 1.585). This means that 4-HPA and PAA concentrations between 31.25 μM and 3mM can be detected dose-responsely with pCMPG10652/TOP10. For 4-HPA only 2–4 hours of incubation is needed, while PAA requires 6–8 hours of incubation for a dose-response detection of 4-HPA or PAA concentrations between 31.25 μM and 3 mM. pCMPG10652/TOP10 does not produce any GFP in response to IAA, which was also observed for the *lacZ* reporter system (section 3.3.1)

3.3.3 Pyocyanin as reporter system

Auxin responsive protein HpaA is able to induce a dose-response production of GFP. The GFP reporter system offers a rapid signal transduction from auxin detection to concentration determination via optical signal detection. The signal in the novel pyocyanin reporter system is transduced to an electrical current. Althought the cellular transduction of the signal in the cell is more complex than that of GFP, the measurement of pyocyanin should offer a rapid signal transduction which can easily be adapted to *in situ* applications.

Pyocyanin

Pseudomonas aeruginosa (*P. aeruginosa*) produces several virulence factors to establish chronic respiratory infections. One of these factors is toxin pyocyanin, a redox-active blue pigment that is required for full virulence. Pyocyanin is associated with airway infections and cystic fibrosis in humans. It plays a key role in several important cellular functions in host cells including immune mechanisms, cellular respiration and electron transport [79, 158].
Pyocyanin is a phenazine, which is a nitrogen-containing heterocyclic compound produced by some bacteria such as *Pseudomonas* and *Streptomyces* species [197]. Phenazines are synthesized from chorismic acid, an intermediate from the shikimic acid pathway. The biosynthesis of pyocyanin from chorismic acid in *P. aeruginosa* is complex and consists of two homologous operons (*phzA1B1C1D1E1F1G1* and *phzA2B2C2D2E2F2G2*) to produce phenazine-1-carboxylic acid (PCA), and two additional genes (*phzM* and *phzS*) whose gene products convert PCA to 5-methylphenazine-1-carboxylic acid (5-MCA) and 5-MCA to pyocyanin respectively (Fig. 3.15) [122].
Pyocyanin oxidizes when a voltage of -240 mV is applied to it, opposed to a

Ag/AgCl electrode. Electrons are exchanged during oxidation, this induces a measurable current equivalent to the concentration of pyocyanin present. More electrons will be exchanged in the oxidation process of a high concentration of pyocyanin than during oxidation of a low concentration of pyocyanin. Hence, a high concentration of pyocyanin will induce a higher current than a low concentration of pyocyanin [17]. While many biological relevant molecules oxidize when a voltage is applied, most oxidize at positive voltages. At negative voltages only phenazines will be detected [215]. Therefore pyocyanin is an interesting candidate for use as a bacterial output product because there is only a small chance of false positives.
In this work, the production of pyocyanin will be measured by means of cyclic voltammetry (CV) whereby a potentiostat applies a series of voltages, cycling 20 times between -0.5 V and 0.1 V, with a 2 mV step voltage. Since pyocyanin oxidizes when a voltage of -240 mV is applied and is reduced at an applied voltage of -280 mV [215] a current will be produced at these voltages. The results of CV experiments are shown as a voltammogram which shows the measured induced current in function of the applied voltage. Therefore, for pyocyanin, a peak of current will be seen in the voltammogram at an applied voltage of -240 mV and -280 mV. The height of these peaks is dependent on the amount of pyocyanin in the medium because high concentrations of pyocyanin will induce high currents.

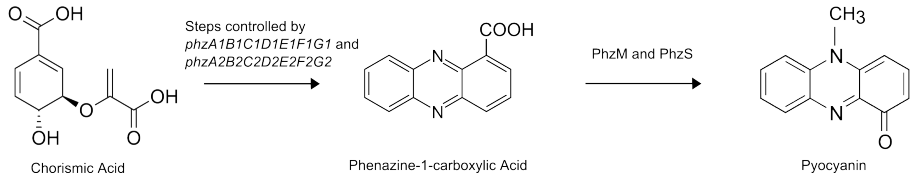


Figure 3.15: Pathway of the production of pyocyanin from chorismic acid. Chorismic acid is converted to PCA by the gene products of the two homologous operons *phzA1B1C1D1E1F1G1* and *phzA2B2C2D2E2F2G2*. PCA is converted to 5-MCA by PhzM from the *phzM* gene. 5-MCA is converted to pyocyanin by PhzS, product of the *phzS* gene.

Design of a pyocyanin-producing auxin bioreporter and biosensor

The pyocyanin-producing auxin bioreporter whose signal can be detected using an electrical readout to produce a biosensor was designed similarly to the GFP-producing bioreporter. Plasmid pUCP-MS was used as template for the output system of the bioreporter. pUCP-MS contains genes *phzM* and *phzS* (Fig. 3.16–B) of *P. aeruginosa* which are needed to convert PCA to 5-MCA

and 5-MCA to pyocyanin respectively [122].
The DNA sequence of pRA_2 (Fig. 3.16–A) containing the constitutive P_A promoter, the *hpaA* gene and 4-HPA responsive promoter P_{BC} was amplified and placed upstream of the *phzM* gene in plasmid pUCP-MS to result in pCMPG10653 (Fig. 3.16–C), as described in materials and methods (chapter 2).
An additional plasmid is needed to complement pCMPG10653 in the construction of a working pyocyanin-producing bioreporter since precursor PCA is usually not readily available in *E. coli* TOP10 cells. Plasmid pCMPG10602 contains the operon comprising genes *phzA2B2C2D2E2F2G2* of *P. aeruginosa* under control of a P_{BAD} promoter [208]. The gene products of this operon convert chorismic acid to PCA and their expression is induced by the presence of arabinose, thus high doses of PCA will be produced when arabinose is present. Figure 3.17 shows a schematical overview of the pyocyanin-producing bioreporter.

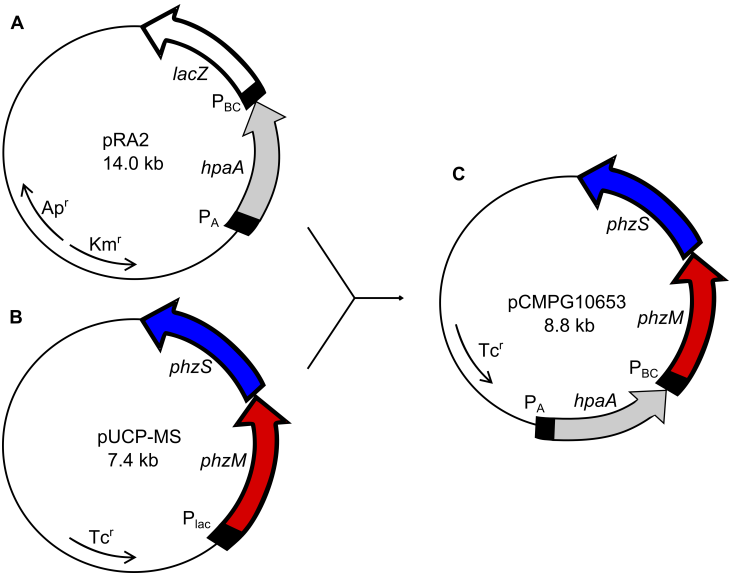


Figure 3.16: Construction of plasmid pCMPG10653. Constitutive P_A promoter, the *hpaA* gene and 4-HPA responsive promoter P_{BC} were copied from plasmid pRA_2 and placed upstream of the *phzM* gene in pUCP-MS. A. pRA_2 B. pUCP-MS C. pCMPG10653

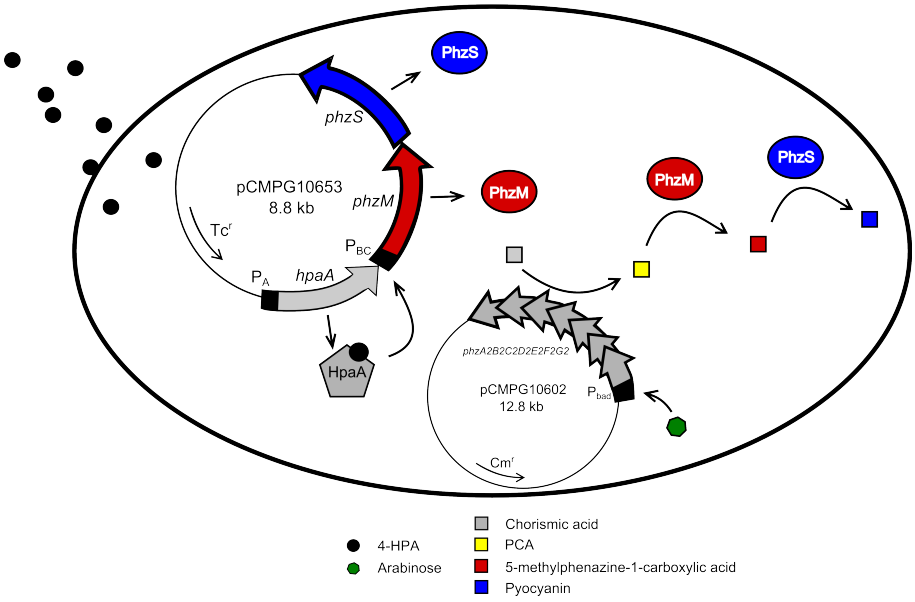


Figure 3.17: Schematical overview of the pyocyanin-producing bioreporter. In the presence of 4-HPA, PhzM and PhzS are expressed. In the presence of arabinose, pCMPG10602 expresses proteins to convert chorismic acid to phenazine-1-carboxylic acid (PCA). Protein PhzM converts PCA to 5-methylphenazine-1-carboxylic acid (5-MCA). Protein PhzS converts 5-methylphenazine-1-carboxylic acid to pyocyanin.

4-HPA and PAA induce a dose-equivalent production of pyocyanin

E. coli TOP10 bacteria were transformed with both pCMPG10602, to convert chorismic acid to PCA, and pCMPG10653, to convert PCA to pyocyanin in the presence of auxins. These bacteria were then grown in the presence of arabinose and either 4-HPA, PAA or IAA. Subsequently, the production of pyocyanin in response to the different auxins was measured by means of CV. The voltammograms of measurements of bacteria containing pCMPG10602 and pCMPG10653 in response to different concentrations of 4-HPA are superimposed in figure 3.18–A whereby the oxidation peak at -240 mV of each coloured graph represents the amount of pyocyanin produced in response to a certain concentration of 4-HPA. The figure shows that a 4-HPA concentration of 15.63 μM elicits the highest amount of pyocyanin production because the current peak at -240 mV is highest for the green voltammogram. Concentrations of 4-HPA above 15.63 μM induced production of pyocyanin but not in a dose-response

manner. Subsequent lower concentrations of 4-HPA elicit lower amounts of pyocyanin production. Figure 3.18–B is a plot of the induced current at an applied voltage of -240 mV in function of the binary logarithm of 4-HPA concentration, thus showing the height of the peak of pyocyanin oxidation for each 4-HPA concentration. The figure corroborates the dose-response relationship of the pyocyanin production in response to 4-HPA concentrations between 1.95 μM and 15.63 μM (binary logarithmic values: -9– -6).
Figure 3.19–A is a superimposition of the voltammograms of the different amounts of pyocyanin produced in response to PAA concentrations from 15.63 μM to 125 μM (binary logarithmic values: -6– -3). As with 4-HPA, an oxidation peak can be seen at -240 mV and an inverted reduction peak at -280 mV which means that PAA also induces the expression of pyocyanin in this auxin biosensor. A PAA concentration of 125 μM elicits the highest amount of pyocyanin production because the current peak at -240 mV of the black voltammogram is higher than the others. Figure 3.19–B is a plot of the induced current at an applied voltage of -240 mV in function of the binary logarithm of PAA concentration. This figure clearly shows that the production of pyocyanin in response to PAA is dose-responsive. A higher concentration than 125 μM of PAA induces pyocyanin production but not in a dose-response manner, similar to pyocyanin production in response to 4-HPA.
Figure 3.20–A is a superimposition of the voltammograms of the different amounts of pyocyanin produced in response to IAA concentrations from 0 M to 500 μM. There was no discernible difference in pyocyanin production between different IAA concentrations. This is shown clearly in figure 3.20–B where the measured current is actually decreasing with an increasing concentration of IAA. IAA therefore elicited no measurable production of pyocyanin at any concentration.

Figures 3.18–A, 3.19–A and 3.20–A all show an additional peak of oxidation at $\pm$ -50 mV and reduction at $\pm$ -100 mV. These peaks cannot be attributed to the oxidation or reduction of pyocyanin. Hence, a different redox-active compound is being produced, the identity of which could not be determined.

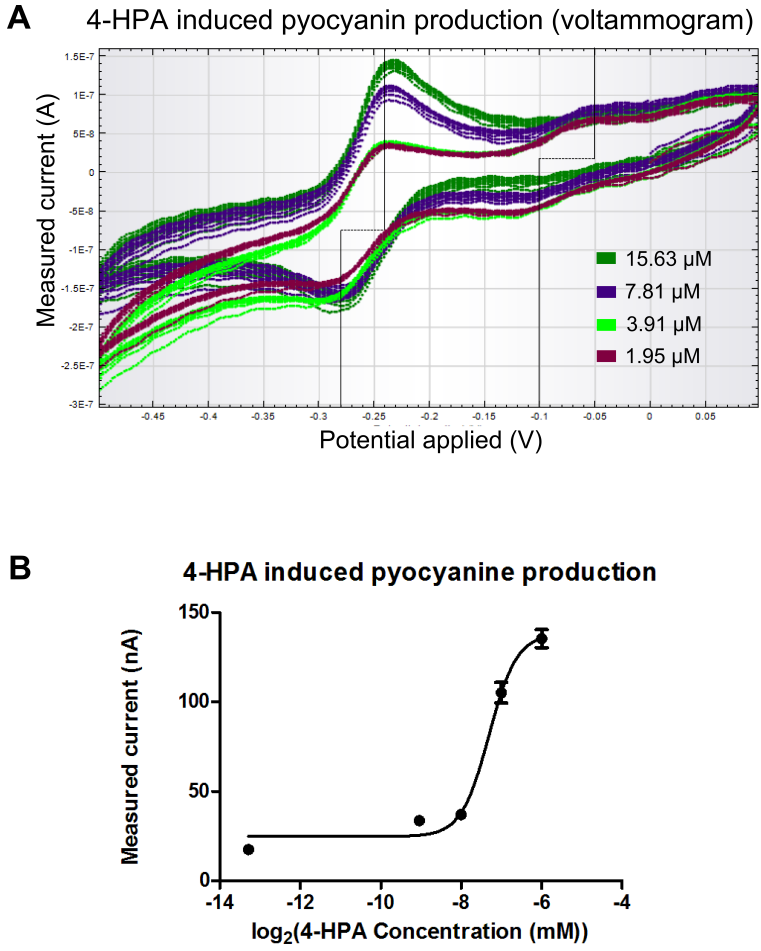


Figure 3.18: A. Superimposition of voltammograms showing the production of pyocyanin in response to 4-HPA (concentrations purple: 1.95 μM, light green: 3.91 μM, blue: 7.81 μM, green: 15.63 μM). Added lines at -240 mV and -280 mV show the peaks representing the production of pyocyanin, added lines at -50 mV and -100 mV show the peaks representing the unknown redox-active compound. B. Plot of induced current at an applied voltage of -240 mV in function of 4-HPA concentration (concentrations 0 M, 1.95 μM, 3.91 μM, 7.81 μM, 15.63 μM). Error bars show standard deviation, based on one biological replicate. The data was fitted using a sigmoidal dose-response fit ($R^2 = 0.9828$).

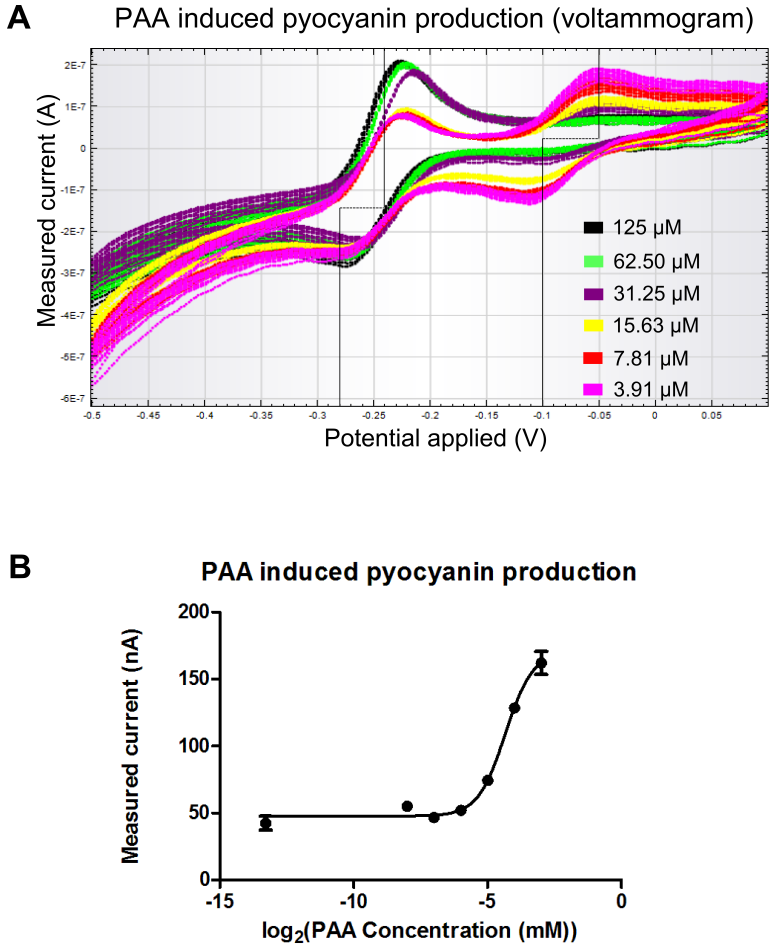
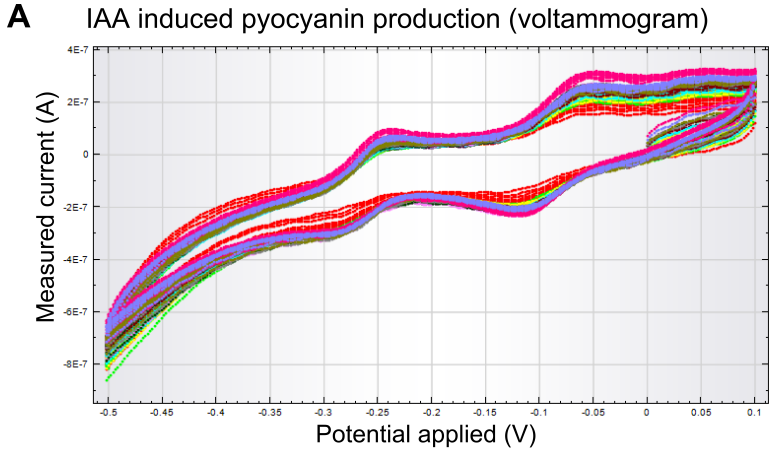


Figure 3.19: A. Superimposition of voltammograms showing the production of pyocyanin in response to PAA (concentrations pink: 3.91 μM, red: 7.81 μM, yellow: 15.63 μM, purple: 31.25 μM, light green: 62.50 μM, black: 125 μM). Added lines at -240 mV and -280 mV show the peaks representing the production of pyocyanin, added lines at -50 mV and -100 mV show the peaks representing the unknown redox-active compound. B. Plot of induced current at an applied voltage of -240 mV in function of PAA concentration (concentrations 0 M, 3.91 μM, 7.81 μM, 15.63 μM, 31.25 μM, 62.50 μM, 125 μM). Error bars show standard deviation, based on one biological replicate. The data was fitted using a sigmoidal dose-response fit ($R^2 = 0.9848$).



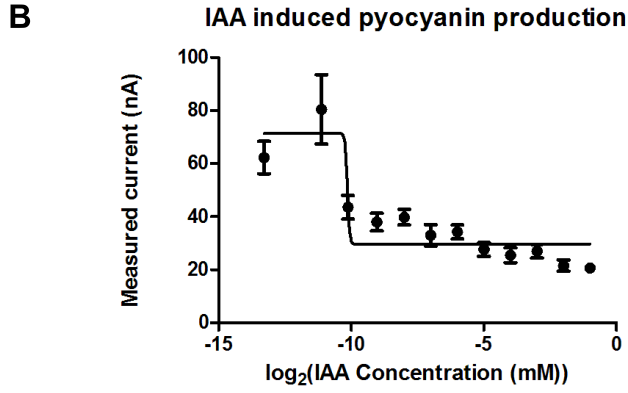


Figure 3.20: A. Superimposition of voltammograms showing the production of pyocyanin in response to IAA B. Plot of induced current at an applied voltage of -240 mV in function of IAA concentration (concentrations 0 M, 0.45 μM, 0.9 μM, 1.95 μM, 3.91 μM, 7.81, μM, 15.63 μM, 31.25 μM, 62.50 μM, 125 μM, 250 μM, 500 μM). Error bars show standard deviation, based on one biological replicate. The data was fitted using a sigmoidal dose-response fit ($R^2 = 0.7798$).

3.4 Discussion

Table 3.2: List of constructed bioreporters described in this chapter.

Plasmid	Function
pCMPG10602	Production of enzymes that convert chorismic acid into PCA
pCMPG10652	Production of GFP in the presence of 4-HPA and PAA
pCMPG10653	Production of pyocyanin in the presence of 4-HPA and PAA

In this chapter, two different whole-cell bacterial auxin bioreporters were designed and constructed. In both cases, the auxin responsive protein HpaA was used as an input module. For the construction of the first bioreporter, HpaA was cloned upstream of a GFP output module, resulting in a fluorescent bioreporter. For the construction of the second, HpaA was combined with a pyocyanin output module, which resulted in an electrochemical biosensor.
The GFP-producing auxin bioreporter (bacteria transformed with pCMPG10652) displays a dose-response result in response to 4-HPA and PAA concentrations from 31.25 μM to 3 mM. The pyocyanin-producing auxin bioreporter (bacteria transformed with pCMPG10602 and pCMPG10653) shows a dose-response production of pyocyanin in response to 4-HPA concentrations from 1.95 μM to 15.63 μM and PAA concentrations from 15.63 μM to 125 μM.

According to Machakova *et al.*, auxins are in most cases active in the concentration range of 0.1–10 μM. Moreover, concentrations higher than 10 μM are often inhibitory [114]. With the exception of the pyocyanin-producing bioreporter that can detect 4-HPA partially in this range, the constructed bioreporters are not sensitive enough to detect active concentrations of auxins. Several limitations contribute to the lower sensitivity of the auxin bioreporters. The most important limitation of the constructed auxin bioreporters is the transport over the cellular membrane. Auxins are lipophilic weak acids and should as such be unable to freely enter bacterial cells by passive membrane diffusion [132]. This appears to be the case for IAA [132] and might be one of the reasons the auxin bioreporters are unable to detect IAA. Prieto and García report that 4-HPA and PAA are able to enter bacterial cells by passive membrane diffusion in concentrations corresponding to the concentrations the auxin bioreporters described in this work were able to detect [156]. However, Prieto and García identified a 4-HPA transport gene, the *hpaX* gene, that codes for a transport protein that actively transports 4-HPA over the bacterial membrane. This could allow for detection of 4-HPA in the concentration range of 0.1–1 μM [156]. The upper limit of 1 μM is lower than the upper detection limit in absence of transport protein (3 mM) because 4-HPA accumulates faster in the bacterial cell as it is transported faster and not metabolized. The use of a transporter protein to actively transport 4-HPA into the bioreporter would also

reduce the time required for a dose-response production of reporter molecule [156]. As of now, no such transporter proteins have been found for either PAA or IAA [132, 183].

The use of arabinose for the induction of the *phzA2B2C2D2E2F2G2* operon, whose gene products are needed to convert chorismic acid to PCA, poses another limitation. The response of the arabinose–P_{BAD} system in individual cells to arabinose concentrations is not linear [89, 176]. *E. coli* cells containing the *gfp* gene under P_{BAD} control display all-or-none behaviour whereby a cell either produces no GFP or a lot of GFP. This was attributed to the need for transporters to transport arabinose over the bacterial membrane. Because cells that are able to transport arabinose into the cell are able to induce the expression of more transporter, resulting in even more arabinose inside the cell and even higher expression of the transporter. The cells that are not able to transport arabinose into the cell on the other hand are unable to induce expression of either the transporter or GFP [90]. It would therefore be useful to replace the P_{BAD} promoter controlling the *phzA2B2C2D2E2F2G2* operon with a constitutive promoter or even the promoter of the *lac* operon which is inducible by the synthetic isopropyl-β-D-thiogalactoside (IPTG) which does not require a transporter to enter the cell.

Comparing both auxin bioreporters, it can be concluded that the GFP-producing auxin bioreporter is able to detect a broader range of concentrations, and higher concentrations, of both 4-HPA and PAA. This difference in sensitivity is likely caused by the greater complexity of the pyocyanin reporter system. While the GFP reporter system consists of a single gene, the pyocyanin reporter system consists of two operons. Both operons encode several enzymes that convert intermediairies to produce one end product. The production of the end product can easily be blocked or delayed in such a long chain of conversions.

Both auxin bioreporters are specific to 4-HPA and PAA since they fail to adequately detect IAA. The amount of β-galactosidase, GFP or pyocyanin produced in response to any concentration of IAA is the same as the production of these reporters in absence of auxins. Besides the fact that IAA is worse at passing the bacterial membrane, figure 1.6 (page 17) shows that IAA differs sufficiently in structure from PAA and 4-HPA to explain that HpaA is unable to bind IAA. All other natural auxins have a structure similar to IAA (Fig. 1.6), but further research is needed to sufficiently determine the selectivity of the bioreporters. The recent characterization of the *iac* genes, coding for enzymes that act in the degradation of IAA can aid in the development of a bioreporter for IAA and the auxins closely resembling IAA [170].
Throughout this chapter PAA has shown to elicit less production of reporter molecule than 4-HPA. This is to be expected since 4-HPA is the natural ligand of HpaA and although PAA closely resembles 4-HPA in structure, the lack

of hydroxyl group prevents PAA from binding to HpaA as efficiently as 4-HPA. Furthermore, PAA can pass the bacterial membrane less easily than 4-HPA [156], this possibly contributes to the lower production of reporter molecules. Incorporating transporter proteins to actively transport both 4-HPA and PAA would improve the detection of both auxins and the time needed for this detection. Additionally, a directed evolution approach or a computer assisted approach could improve HpaA to better bind PAA and help to create bioreporters (more) specific to 4-HPA and PAA.

The additional peak of oxidation at -50 mV observed in figures 3.18–A, 3.19–A and 3.20–A cannot be attributed to the presence of pyocyanin. The height of the additional peak is inversely related to the concentration of auxin whereby low concentrations show a higher peak at -50 mV. Therefore another redox-active molecule is being produced in the presence of low amounts of auxins, even in the presence of IAA, which does not induce expression of pyocyanin itself. This molecule is likely also a phenazine or a precursor since it oxidizes at a negative voltage, but could not be identified yet. There are a number of possible candidates for this phenazine since the conversion of chorismic acid to pyocyanin has many intermediates. The most likely candidate is PCA because this intermediate is produced continuously by the products of plasmid pCMPG10602. PCA is converted to pyocyanin by PhzM and PhzS, but at low auxin concentrations the expression of PhzM and PhzS will also be low. Thus the amount of PCA will build up because it cannot be converted to pyocyanin. This would also explain the peak at -50 mV for IAA. Nevertheless, the oxidation of PCA happens at -400 mV [17] and not at -50 mV. The only intermediate which oxidizes at approximately -50 mV is 5-MCA whose oxidation happens at -70 mV and reduction at -100 mV [17]. 5-MCA is thus very likely the intermediate phenazine that is visible on the voltammograms. 5-MCA is converted from PCA by PhzM and is normally converted to pyocyanin by PhzS. This might suggest that the expression of *phzS* is lower than that of *phzM* at low concentrations of auxins. Here too, further research is needed to determine the source of the accumulating molecule.
In the genome of *P. aeruginosa*, *phzM* and *phzS* are located on opposite ends of operon *phzA1B1C1D1E1F1G1*, both with their own promoter. In pUCP-MS, both genes were placed under control of the promoter of the *lac* operon. pUCP-MS was originally constructed to study the production of pyocyanin and might therefore not be suitable to use as a reporter system.

In summary, the auxin bioreporter's production of three different reporter systems (LacZ, GFP and pyocyanin) was measured in response to three different auxins (4-HPA, PAA and IAA).
The β-galactosidase producing auxin bioreporter, pRA_2/TOP10, can detect 4-HPA and PAA, up until a concentration of 1 mM. However, the *lacZ* reporter

system is labour-intensive and difficult to use *in situ*.
The GFP-producing auxin bioreporter, pCMPG10652/TOP10, can detect 4-HPA and PAA in a broad range of concentrations with a detection limit of 31.25 μM.
The pyocyanin-producing auxin biosensor, pCMPG10602&pCMPG10653/TOP10, is able to detect 4-HPA and PAA at low concentrations but only between limited concentrations. All three however, are unable to detect IAA at any concentration.

Chapter 4

Design and construction of steroid hormone bioreporters

4.1 Abstract

The increased use of oral contraceptives is a risk to worldwide water safety. To estimate the severity of the danger, an adequate sensor for steroid hormones is required. As of now, the only available methods for the detection of steroid hormones are labour-intensive, expensive methods. There is a need for a fast, cost-effective, *in situ* sensor. In an effort to provide such a sensor, this chapter describes the design and construction of two different whole-cell bacterial steroid hormone bioreporters. Both use the steroid hormone responsive protein RepA combined with the two operator sites it binds to, as an input module. For the first steroid hormone bioreporter, the signal from the input module is relayed to the *gfp* gene as output module, effectively creating a fluorescent bioreporter. The second bioreporter relays the input signal to the genes *phzM* and *phzS* to produce the redox-active molecule pyocyanin. The output signal of this bioreporter is measured electrochemically and can be integrated with a MEA, resulting in a whole-cell biosensor.
The fluorescent bioreporter is able to dose-responsely detect the presence of testosterone, 17β-estradiol, 17α-ethinylestradiol, estrone and progesterone with detection limits between 250 μM and 1 mM.
The pyocyanin-producing bioreporter is unable to produce pyocyanin in the presence of steroid hormones.

4.2 Introduction

The steroid hormone reporters are whole-cell bacterial bioreporters. Similar to the auxin bioreporters (chapter 3), they consist of an input module and two different output modules. The input module consists of a steroid responsive protein, transcriptional repressor RepA, which is found in the testosterone-degrading bacteria *Comamonas testeroni* (*C. testosteroni*). RepA transduces the presence of steroid hormones by releasing two operator DNA sequences and freeing up a promoter, this activates the expression of the output module.
In this chapter the design and construction of two whole-cell bacterial steroid hormone bioreporters is described. First, some background on bacterium *C. testosteroni* is provided in section 4.2.1. This is followed by a description of the role of steroid responsive repressor RepA in the regulation of the expression of 3α-hydroxysteroid dehydrogenase/reductase (3α-HSD/CR) in section 4.2.2. Next, section 4.3.1 shows the design and experimental results of a steroid hormone bioreporter using GFP as reporter system, followed by the results of a bioreporter using pyocyanin as reporter system in section 4.3.2. And finally, this chapter ends with a discussion of the results of both bioreporters in section 4.4.

4.2.1 *Comamonas testosteroni*

C. testosteroni are Gram-negative soil bacteria. They are motile, nonspore-forming rods that possess one to five polar flagella [187]. *C. testosteroni* can be found in soil and water environments where they live under strictly aerobic conditions [53]. In very rare cases, the bacterium has been found to infect humans [2, 30, 53].
All members of the *Comamonas* genus can assimilate a variety of organic compounds but none use sugars as carbon source [187], *C. testosteroni*, for example, survives on testosterone metabolism. As such, it is often found in active sludge of sewage treatment plants where it increases the breakdown of aromatic hydrocarbons like steroids [23, 82, 105]. Active sludge is part of the process for treating sewage and industrial wastewaters and consists of a hugely diverse population of bacteria [83]. Its main purpose is to oxidize biological matter and to remove phosphates from wastewater. *C. testosteroni* in active sludge breaks down steroids by making use of short-chain dehydrogenase/reductases (SDR). This large superfamily of NAD (P)(H)-dependent oxidoreductases includes enzymes like 3α-HSD/CR which catalyzes the oxidation at position three of the steroid nucleus to start the breakdown of steroids [125] (Fig. 4.1).
The expression of 3α-HSD/CR in *C. testosteroni* is regulated by steroid responsive repressor RepA.

4.2.2 Regulation of 3α-hydroxysteroid dehydrogenase/reductase expression

The regulation of *3α-hsd*, the gene encoding 3α-HSD/CR is shown schematically in figure 4.1. The *3α-hsd* gene is preceded by two operator sites, 10 nucleotide palindromic sequences separated by approximately 1600 nucleotides. Repressor RepA, product of gene *repA*, binds to the two operator sites in the absence of steroid hormones. By binding to the operator sites, a DNA loop is formed which prevents RNA polymerase from binding to *3α-hsd*'s promoter. Steroid hormones present in the environment bind RepA causing it to release the operator sites. This in turn frees *3α-hsd*'s promoter for RNA polymerase to start the transcription of 3α-HSD/CR [220].

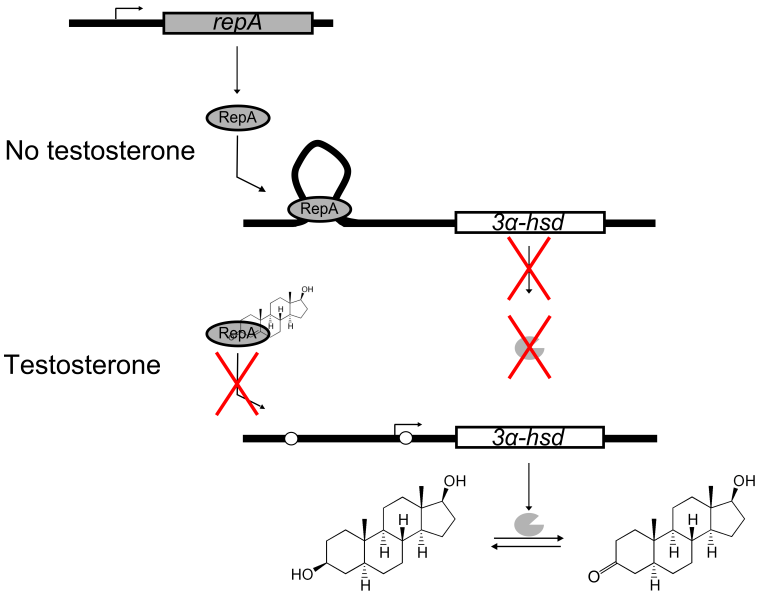


Figure 4.1: Regulation of the expression of *3α-hsd*. Repressor RepA binds to two operator sites upstream of *3α-hsd* in the absence of steroid hormones. This causes the creation of a loop which prevents RNA polymerase from binding to *3α-hsd*'s promoter. In the presence of steroid hormones, RepA will release the operator sites after binding steroid hormones. This frees the promoter of *3α-hsd* for RNA polymerase and causes the expression of 3α-HSD/CR .

Maser and Xiong used this mechanism to construct the COSS (as described in section 1.5) [121]. This system was constructed by placing the *gfp* gene into the chromosome of *C. testosteroni* by homologous integration. This resulted in a rapid and easy to manipulate steroid sensoring system that is sensitive (17β-estradiol concentrations as low as 1.6 mg/L can be detected and even as low as 29 pg/L in a cell-free setup). However, *C. testosteroni* is not well known compared to bacteria like *E. coli*, including its ideal culturing conditions. Additionally, *E. coli* is the organism of preference for the construction of bioreporters. For this reason, the bacterial steroid hormone bioreporter, described in this chapter, was constructed using *E. coli* as chassis, with *C. testosteroni*'s repressor RepA and the two operator sites it binds to as input for the bacterial bioreporter. This was combined with two different output systems, GFP and pyocyanin, to produce two different whole-cell steroid hormone bioreporters.

4.3 Results

4.3.1 GFP as reporter system

Design of a GFP-producing steroid hormone bioreporter

Figure 4.2 shows the part of the *C. testosteroni*'s genome containing the *3α-hsd* gene and its regulatory system. The most notable aspect of this DNA sequence is the fact that the *repA* gene largely overlaps with both the *3α-hsd* gene and the second operator site. Thus, simply replacing gene *3α-hsd* with the *gfp* gene would impair the *repA* gene. Since RepA represses the expression of GFP in the absence of steroid hormones, an impaired *repA* gene would ensure expression of GFP regardless of the presence or absence of steroid hormones. This would defeat the purpose of the bioreporter. In the construction of COSS, the *repA* gene was essentially moved downstream to make room for the *gfp* gene in the *C. testosteroni* chromosome. A similar but different strategy was used for the construction of a steroid hormone bioreporter described in this chapter.

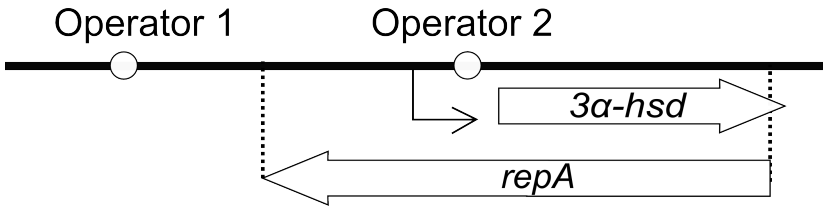


Figure 4.2: Close up of a part of the *C. testosteroni* genome. Operator 1 and operator 2 are separated by approximately 1600 nucleotides. Gene *3α-hsd*'s promoter overlaps with operator 2. The *repA* gene overlaps with operator 2 and the *3α-hsd* gene but is oriented in the opposite direction.

To remove the overlap, the promoter and the two operator sites were split from the *repA* gene and cloned in two separate plasmids (Fig. 4.3). Plasmid pCMPG10654 is constructed by cloning the promoter and two operator sites of *C. testosteroni*, in vector pFPV25 upstream of the *gfp* gene. Plasmid pCMPG10658 is constructed by cloning the *repA* gene downstream of the arabinose inducible P_{BAD} promoter in vector pBAD332. Bacteria transformed with both plasmids should therefore express RepA in the presence of arabinose, GFP in the absence of arabinose and, RepA and GFP in the presence of both arabinose and steroid hormones. To confirm whether this indeed is the expression pattern of the bioreporter, fluorescence measurements of bacteria carrying both plasmid pCMPG10654 and pCMPG10658 were performed, the

results of which can be seen in figure 4.4. In the absence of arabinose there is no expression of repressor RepA, as a consequence the expression of GFP cannot be repressed and a high measured fluorescence intensity is measured. In the presence of 0.02% arabinose, there is expression of repressor RepA. RepA binds to the two operator sites upstream of the *gfp* gene and represses the expression of GFP resulting in a significantly lower measured fluorescence intensity. In the presence of both arabinose and 250 μM testosterone, repressor RepA is expressed and represses the expression of GFP. However, testosterone in the medium binds to RepA after which RepA releases the two operator sites and expression of GFP is resumed. Although bacteria containing both plasmid pCMPG10654 and pCMPG106858 show an increase in GFP expression in the presence of 0.02% arabinose and 250 μM testosterone, this increase is not statistically significant (Fig. 4.4).

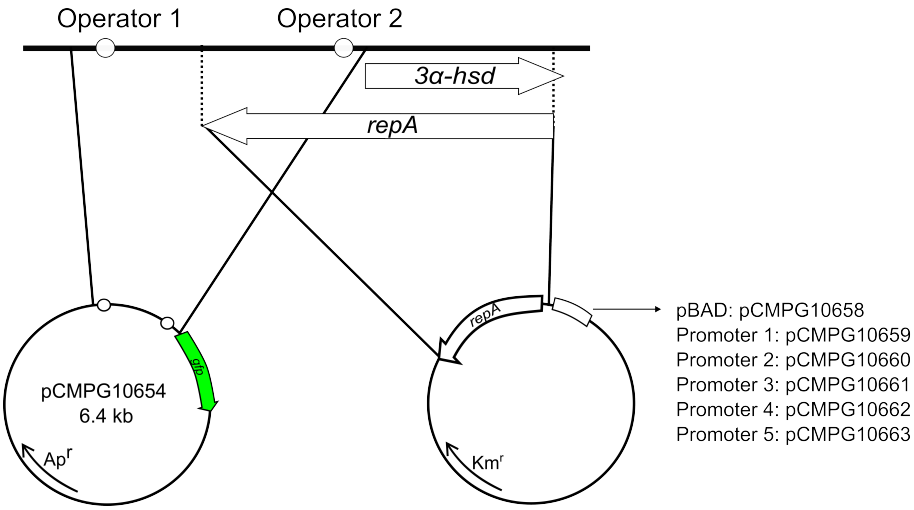


Figure 4.3: Design of plasmids pCMPG10654 and pCMPG10658. The two operator sites of *C.testosteroni* were copied and placed upstream of the *gfp* gene in plasmid pFVP25. This resulted in plasmid pCMPG10654. The *repA* gene of *C. testosteroni* was copied and placed downstream of the P_{BAD} promoter in plasmid pBAD332K, resulting in pCMPG10658.

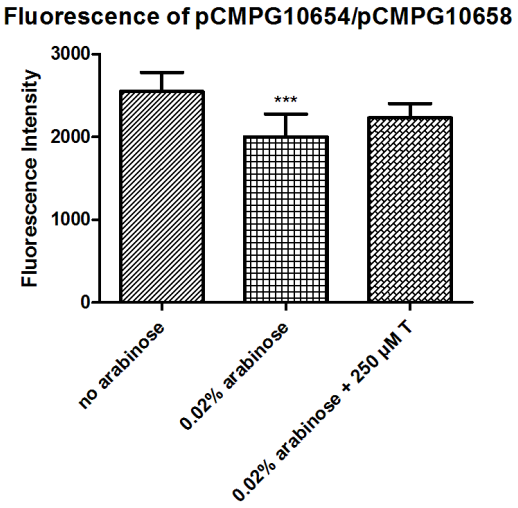


Figure 4.4: Fluorescence produced by bacteria transformed with pCMPG10654 and pCMPG10658–pCMPG10663. In the absence of arabinose, the RepA repressor is not expressed, therefore, there is expression of GFP. In the presence of only arabinose, the RepA repressor is expressed and expression of GFP is significantly lower. In the presence of both arabinose and testosterone, the RepA repressor should release the two operator sites and expression of GFP should follow. However, the increase in fluorescence intensity when testosterone is added is not statistically significant.

Construction of constitutively RepA expressing plasmids

Using plasmids pCMPG10654 and pCMPG10658 results in a bioreporter that has two possible inputs that influence the expression of GFP, namely arabinose and steroid hormones. This reduces reproducibility, even more so because induction by arabinose at subsaturating concentrations is bimodal, which means that *gfp* gene expression under control of a P_{BAD} promoter is not uniform with respect to individual cells [176]. Therefore, a small deviation of arabinose concentration between measurements can give very different results between experiments, this reduces the reproducibility of the bioreporter significantly. To improve on the reproducibility, the expression of RepA was made constitutive. However, the degree of constitutivity, whether the expression is high, medium, low or somewhere in between, is expected to determine the signal-to-noise ratio of the bioreporter. This degree of constitutivity is determined by the strength of the promoter/ribosome binding sites (RBS) combination. A low expression constitutive promoter/RBS combination upstream of the *repA* gene causes the expression of a low amount of RepA to inhibit the expression of GFP. This might give rise to a high amount of leaky GFP expression because of insufficient repression. While a high expression constitutive promoter/RBS pair upstream of the *repA* gene causes the expression of a high amount of RepA inhibiting the expression of GFP to a high degree. But the high amount of RepA repressor might reduce the measurable signal considerably.
Accordingly, five new plasmids were constructed (pCMPG10659-pCMPG10663) by cloning different combinations of constitutive promoters and RBS upstream of the *repA* gene (Table: 4.1). These five plasmids express RepA constitutively, be it to different degrees. A different degree of RepA expression leads to different degrees of GFP repression and subsequently to different signal-to-noise ratios. Fluorescence measurements were performed to determine the optimal level of repression for a maximum signal-to-noise ratio.

Steroid hormones affect both fluorescence intensity and optical density

Five different steroid hormones were used in all experiments in this chapter, they are : testosterone, 17β-estradiol, progesterone, 17α-ethinylestradiol and estrone (Fig. 4.5). Testosterone was chosen because it is the standard ligand for RepA, the others were chosen because of their prevalence in the environment, due to their role in the hormonal cycle.
It was found early on that the addition of the steroid hormones affects both the fluorescence intensity and absorbance (OD_{595}) measurements of the cultures. To determine the extent of the effect of steroid hormones on these measurements, an experimental setup was devised whereby the fluorescence and the OD_{595} of

Table 4.1: Plasmids containing constitutive RBS and promoter combinations upstream of the *repA* gene. Names are taken from the Bioregistry database [179]. The resulting strength of each combination is stated in the strength column. The last column contains the name given to bacteria containing plasmid pCMPG10654 and the specified plasmid.

Plasmid	Promoter	RBS	Strength	Strain name
pCMPG10659	J23100	B0034	Strong	Strong1RepA
pCMPG10660	J23114	B0034	Weak	Weak1RepA
pCMPG10661	J23104	B0032	Weak	Weak2RepA
pCMPG10662	J23110	B0031	Strong	Strong2RepA
pCMPG10663	J23110	B0032	Medium	MediumRepA

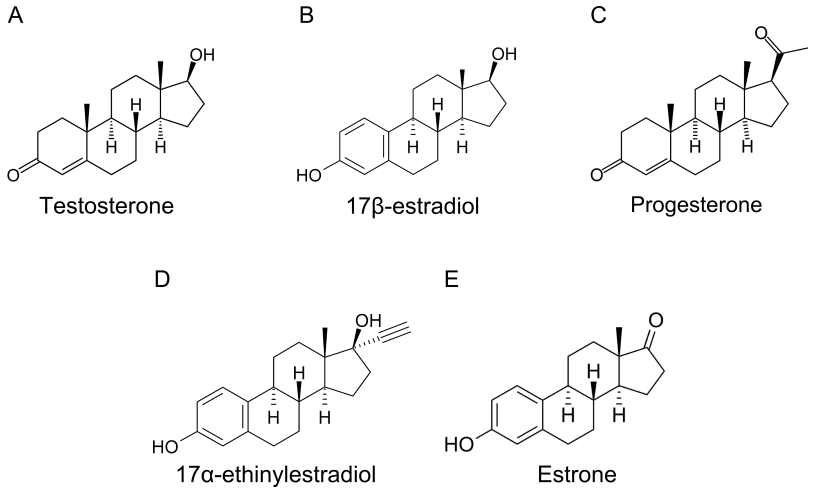


Figure 4.5: Structures of steroid hormones used in this work. A. Testosterone B. 17β-estradiol C. Progesterone D. 17α-ethinylestradiol E. Estrone

bacteria containing pFPV25.1 were measured in the presence of a dilution series of testosterone from 30 μM to 3 mM (as described in section 2.4). Plasmid pFPV25.1 is a derivative of pFPV25 with the constitutive promoter rpsM upstream of the *gfp* gene. The constitutive production of GFP allows for a quick observation of the effect of steroid hormones on fluorescence intensity. The results of the experiment are shown in figure 4.6.

The left figure shows that the OD_{595} is increased by 0.27 in the presence of 3 mM testosterone. This increase in OD_{595} is lowered with decreasing testosterone

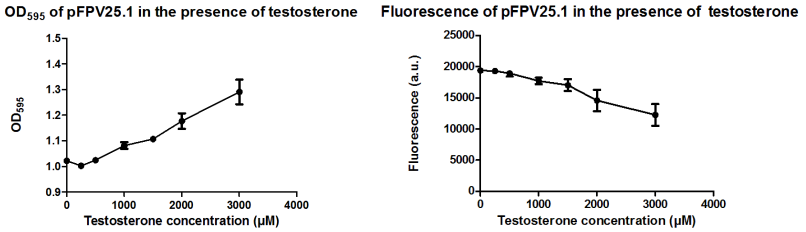


Figure 4.6: Left: OD_{595} Right: Fluorescence intensity of bacteria containing pFPV25.1 in the presence of a concentration series of testosterone.

concentration to the point where 250 μM has a negligible effect on the OD_{595}. The right figure shows that the fluorescence intensity is over 1.6 times lower in the presence of 3 mM testosterone. Here too, concentrations of 250 μM and lower have a negligible effect.
For the analysis of the experiments performed using the fluorescent bioreporter, measured fluorescence intensity is corrected for growth using the measured OD_{595} values. If the measured OD_{595} values are artificially increased and the measured fluorescence intensity is artificially decreased in the presence of steroid hormones, the final results are decreased twice. To compensate for the effect of steroid hormones on both the OD_{595} and the fluorescence intensity, a correction factor was determined experimentally and applied to all results obtained in fluorescence experiments performed with steroid hormones. The determination of this correction factor is explained in section 2.4 at page 41.

GFP is produced dose-equivalently in response to steroid hormones

E. coli TOP10 bacteria were transformed with plasmid pCMPG10654 and either one of pCMPG10659-pCMPG10663, resulting in respectively Strong1RepA, Weak1RepA, Weak2RepA, Strong2RepA and MediumRepA. These bacterial strains should express repressor RepA continuously and have no or a low expression of GFP in the absence of steroid hormones. When steroid hormones are present, a dose-response of GFP expression should be seen. To verify this, all five different strains were grown in the presence of either testosterone, 17β-estradiol, 17α-ethinylestradiol, estrone or progesterone, after which fluorescence measurements were performed. The results are shown in figures 4.7–4.11 where the amount of fluorescence produced is shown in function of the binary logarithm ($\log_2$) of the steroid hormone concentration. All data is fitted using a sigmoidal dose-response fit as described in section 2.6, the R^2-values obtained from these fits are listed in table 4.2 and will be used as an objective standard.

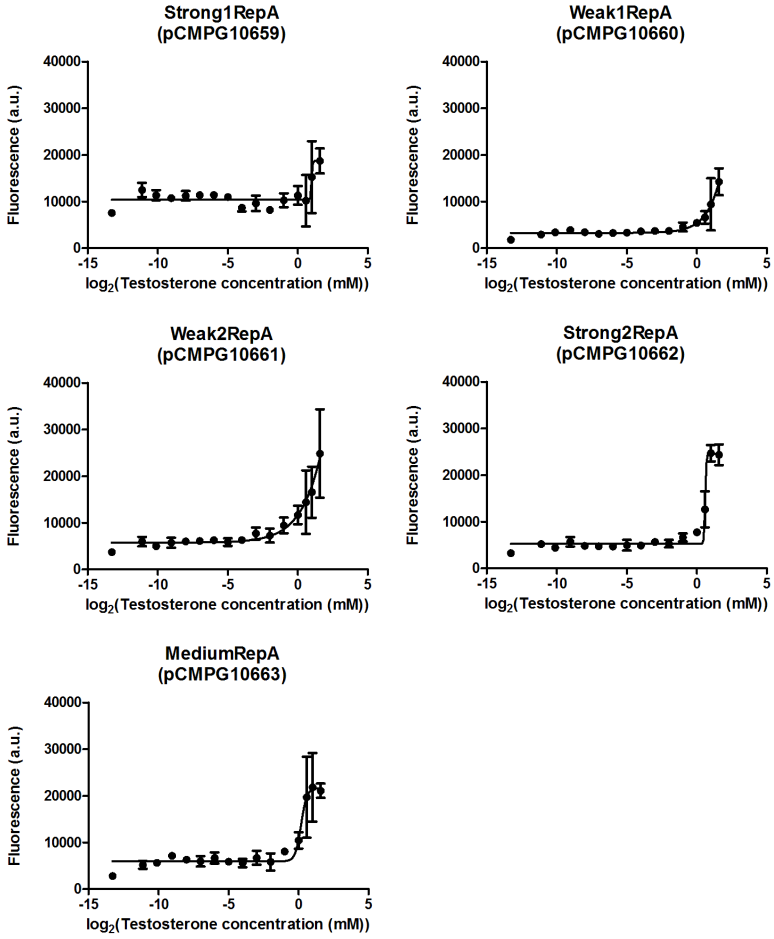


Figure 4.7: Fluorescence produced by bacteria containing pCMPG10654 and either one of pCMPG10659-pCMPG10663 in the presence of testosterone (concentrations 0 M, 0.45 μM, 0.9 μM, 1.9 μM, 3.9 μM, 7.8, μM, 15.625 μM, 31.25 μM, 62 μM, 125 μM, 250 μM, 500 μM, 1 mM, 1.5 mM, 2 mM, 3 mM). Error bars show standard deviation, based on four replicates. The data was fitted using a sigmoidal dose-response fit.

Table 4.2: List of R^2-values obtained from the sigmoidal dose-response fits of the GFP production in response to steroid hormones testosterone, 17β-estradiol, 17α-ethinylestradiol, estrone or progesterone for bacteria transformed with plasmid pCMPG10654 and either one of pCMPG10659-pCMPG10663. The average of the R^2-values for each steroid hormone is shown in the last row. The average of the R^2-values for each constitutive promoter containing plasmid is shown in the last column. Empty boxes denote data for which no fit could be obtained.

	R^2-value					
Strain	**T**	**E2**	**EE2**	**E1**	**P**	**Average**
Strong1RepA	0.4267	0.6661	0.8926	0.6574	0.8184	**0.6922**
Weak1RepA	0.7898	0.7818	0.9069	0.4187	0.9306	**0.7656**
Weak2RepA	0.7542		0.8074	0.8344	0.8638	**0.8149**
Strong2RepA	0.9408	0.8846	0.9627	0.4991	0.9447	**0.8464**
MediumRepA	0.7960	0.6885	0.7419		0.9339	**0.7901**
Average	**0.7415**	**0.7552**	**0.8623**	**0.6024**	**0.8983**	

Figure 4.7 shows the production of GFP of Strong1RepA, Weak1RepA, Weak2RepA, Strong2RepA and MediumRepA in the presence of **testosterone**. All five plasmids are able to produce GFP in response to testosterone, Weak2RepA, Strong2RepA and MediumRepA bacteria show a higher fluorescence intensity than Strong1RepA and Weak1RepA. The R^2-values (table 4.2) show that Strong2RepA bacteria produce the best dose-response production of GFP in the presence of testosterone ($R^2 = 0.9408$). Weak1RepA, Weak2RepA and MediumRepA bacteria also produce GFP dose-responsely, albeit it to a lesser degree than bacteria containing pCMPG10662 (R^2-values are 0.7898, 0.7542 and 0.7960 respectively). However, Weak2RepA posesses the lowest limit of detection, the linear part of the sigmoidal fit starts at 250 μM (binary logarithmic value: -2) and a plateau has not been reached yet. Weak2RepA bacteria can therefore detect concentrations testosterone concentrations from 250 μM and higher. The other bacteria strains have a higher limit of detection.

Figure 4.8 shows the production of GFP produced in the presence of **17β-estradiol**. There is a low production of GFP by all plasmids in response to 17β-estradiol, except for Strong2RepA bacteria which produce a high amount of GFP in response to high concentrations of 17β-estradiol. Strong2RepA also displays the best dose-response production of GFP ($R^2 = 0.8846$). Strong2RepA can detect 17β-estradiol concentrations of 1 mM (binary logarithmic value: 0) and higher, possibly even higher than 3 mM since a plateau has not yet been reached.

The production of GFP in response to **17α-ethinylestradiol** is shown in figure 4.9. In general 17α-ethinylestradiol elicits a low production of GFP while the

corresponding R^2-values are relatively high. Strong2RepA produces a high amount of GFP dose-responsely in the presence of 17α-ethinylestradiol (R^2 = 0.9627). The other strains display a dose-response production of low amounts of GFP. All strains can detect 17α-ethinylestradiol concentrations of 1 mM (binary logarithmic value: 0) and higher, Strong2RepA can possibly detection concentrations above 3 mM as well.

Estrone elicits the lowest amount of GFP production as can be seen in figure 4.10. Moreover, the R^2-values for the experiments performed in the presence of estrone are generally lower. Weak2RepA produces the best dose-response result in the presence of estrone (R^2 = 0.8344). All strains, except for MediumRepA can detect estrone concentrations of 1 mM and higher.

Finally, **progesterone** generally elicits a low amount of GFP production. The only exceptions are Strong2RepA and MediumRepA bacteria, the former of which produces up to six times as much GFP as the other strains. Both Strong2RepA and MediumRepA have a high R^2-value (R^2 = 0.9447 and R^2 = 0.9339 respectively). All strains have a detection limit of 1mM.

In general it can be observed that Strong2RepA bacteria usually show the highest amount of GFP production and the best dose-response result(*i.e.* high R^2-values, average over all hormones is R^2 = 0.8464). Strong1RepA's dose-responseness is the lowest of all. Progesterone and 17α-ethinylestradiol elicit the best dose-response production of GFP (average R^2-values of R^2 = 0.8983 and R^2 = 0.8623 respectively. Estrone elicits the least dose-response production of GFP and the lowest amount of GFP as well.

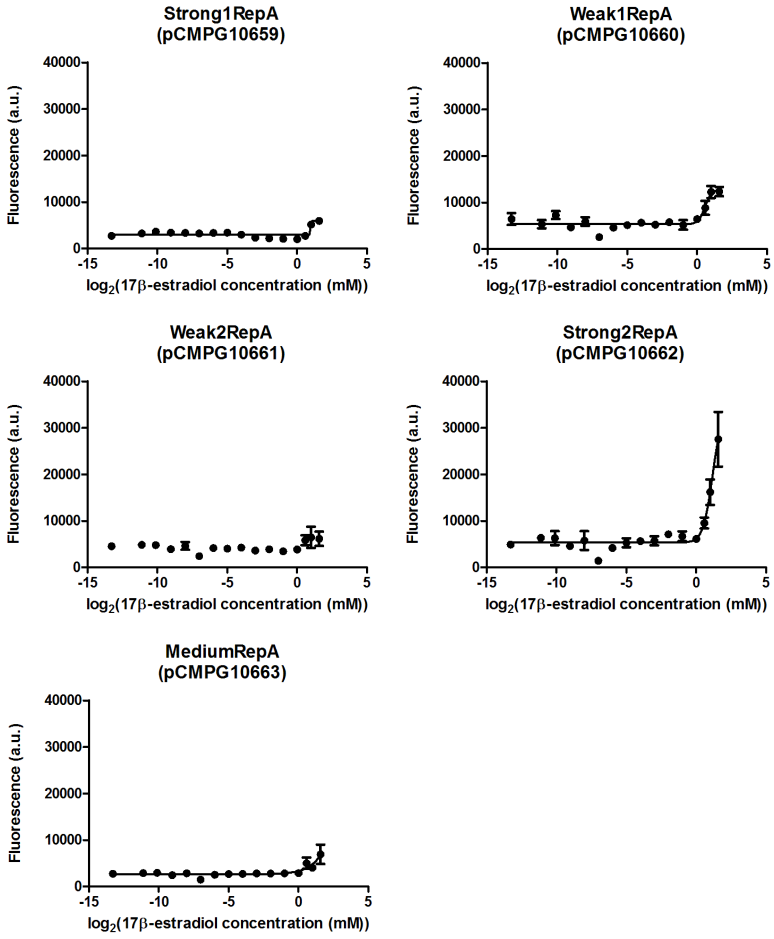


Figure 4.8: Fluorescence produced by bacteria containing pCMPG10654 and either one of pCMPG10659-pCMPG10663 in the presence of 17β-estradiol (concentrations 125 μM, 250 μM, 500 μM, 1 mM, 1.5 mM, 2 mM, 3 mM). Error bars show standard deviation, based on four replicates. The data was fitted using a sigmoidal dose-response fit.

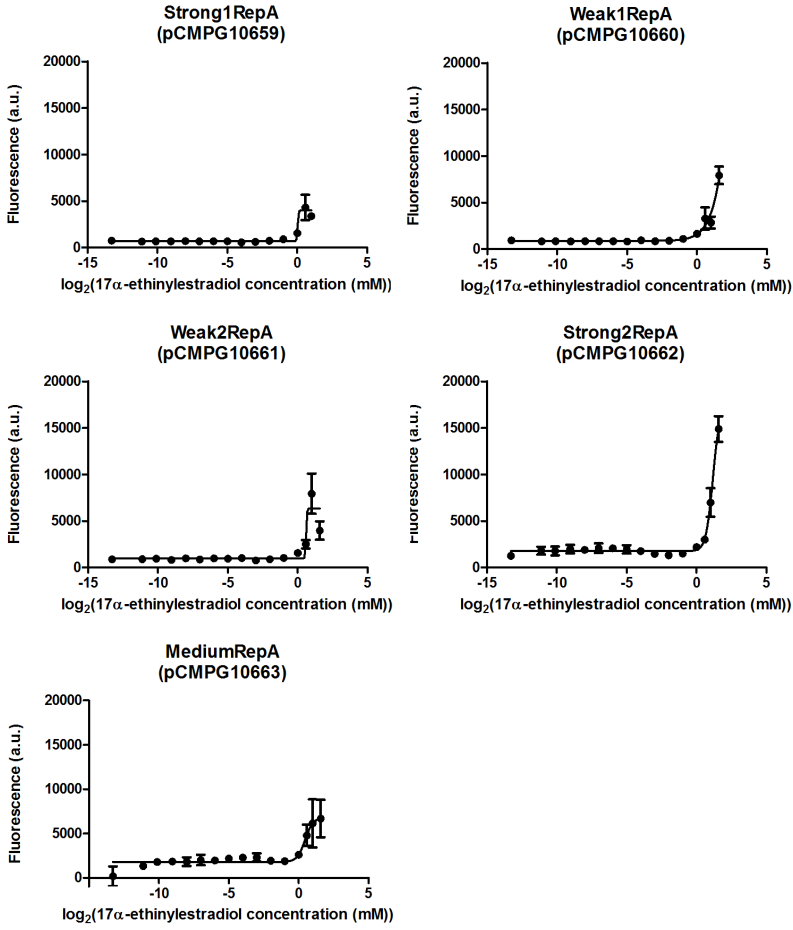


Figure 4.9: Fluorescence produced by bacteria containing pCMPG10654 and either one of pCMPG10659-pCMPG10663 in the presence of 17α-ethinylestradiol (concentrations 125 μM, 250 μM, 500 μM, 1 mM, 1.5 mM, 2 mM, 3 mM). Error bars show standard deviation, based on four replicates. The data was fitted using a sigmoidal dose-response fit.

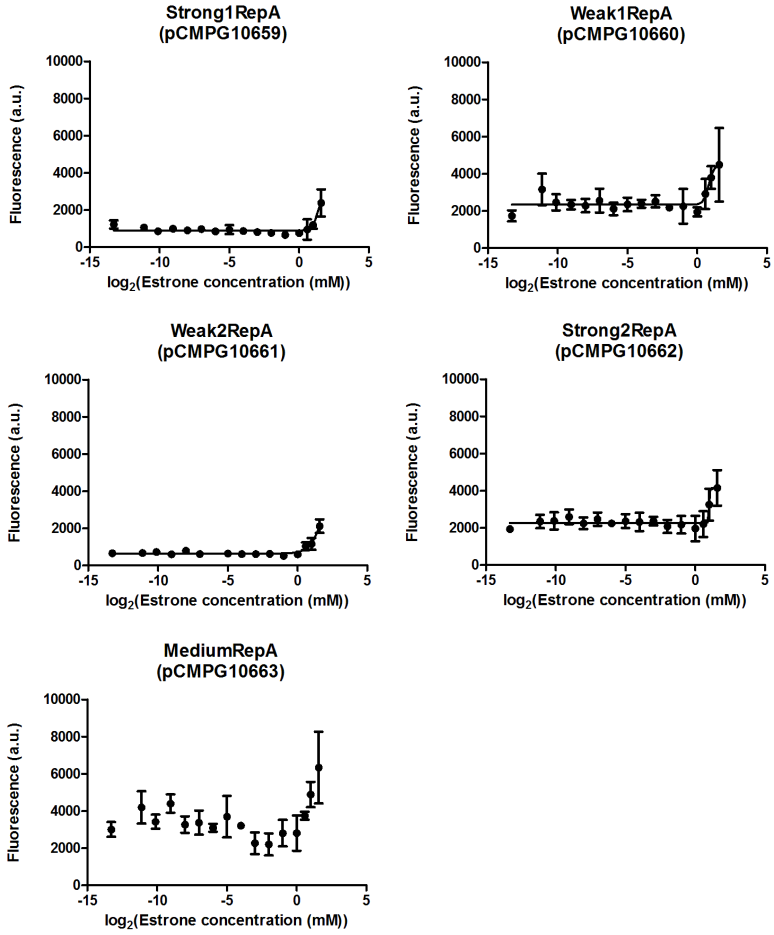


Figure 4.10: Fluorescence produced by bacteria containing pCMPG10654 and either one of pCMPG10659-pCMPG10663 in the presence of estrone (concentrations 125 μM, 250 μM, 500 μM, 1 mM, 1.5 mM, 2 mM, 3 mM). Error bars show standard deviation, based on four replicates. The data was fitted using a sigmoidal dose-response fit.

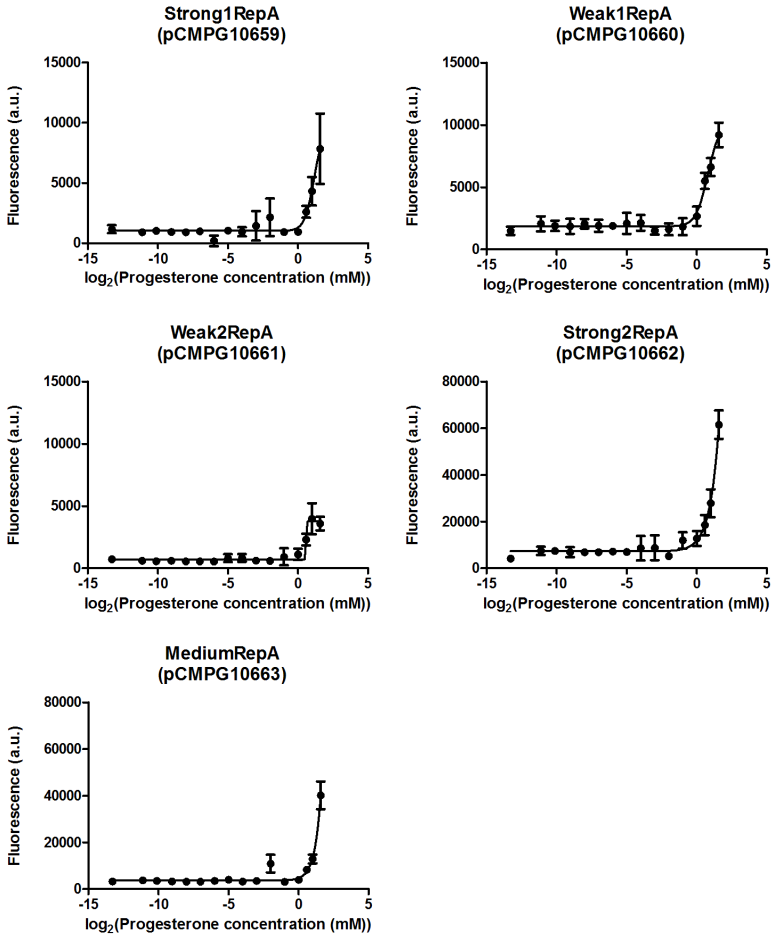


Figure 4.11: Fluorescence produced by bacteria containing pCMPG10654 and either one of pCMPG10659-pCMPG10663 in the presence of progesterone (concentrations 125 μM, 250 μM, 500 μM, 1 mM, 1.5 mM, 2 mM, 3 mM). Strong2RepA and MediumRepA produce substantially more GFP than the other strains. Error bars show standard deviation, based on four replicates. The data was fitted using a sigmoidal dose-response fit.

4.3.2 Pyocyanin as reporter system

Design of a pyocyanin-producing steroid hormone bioreporter

The design of the pyocyanin-producing steroid hormone bioreporter is similar to that of the GFP-producing steroid hormone bioreporter. The DNA sequence of *C. testosteroni* containing the two operator sites was spliced to a DNA fragment consisting of the *phzM* and *phzS* genes of pUCP-MS, upstream of *phzM*. This splice product was cloned into plasmid pBBR1MCS-2 resulting in plasmid pCMPG10657 (Fig. 4.12). Bacteria transformed with this plasmid should express PhzM and PhzS constitutively and therefore convert PCA to pyocyanin. However, bacteria transformed with plasmid pCMPG10657 are unable to convert PCA to pyocyanin. Instead of blue pyocyanin a red pigment is produced.
Figure 4.13 shows a more detailed scheme of the pyocyanin pathway of *P. aeruginosa* to identify the possible source of the red pigment. Chorismic acid is converted to PCA by the gene products of the two homologous operons *phzA1B1C1D1E1F1G1* and *phzA2B2C2D2E2F2G2*. Subsequently, PCA is converted to red 5-MCA by PhzM. 5-MCA in turn is converted to pyocyanin by PhzS. However, PhzS can also convert PCA to 1-hydroxyphenazine (1-OH-PHZ). Both 5-MCA and 1-OH-PHZ are red pigments [52, 122], which means that probably either of these two is produced. If both PhzM and PhzS are expressed, there should be pyocyanin production. This could mean that only PhzM is expressed or only PhzS is expressed since either of these converts PCA to a red-coloured pigment. Plasmid pCMPG10657 was constructed by placing the two operator sites upstream of the *phzM* gene, it is therefore more likely that PhzS is expressed insufficiently or not at all. To have an equal expression of both genes, a second plasmid was constructed whereby the two operator sites were placed upstream of *phzS* as well as upstream of *phzM*, this resulted in plasmid pCMPG10664. Nonetheless, pCMPG10664 too is also unable to convert PCA to pyocyanin, here too a red pigment is produced. The pigment was subjected to chromatographic analysis and while its identity could not be determined, PCA or 1-OH-PHZ have been excluded. This points to 5-MCA as the red pigment, but this is still to be confirmed in future research.

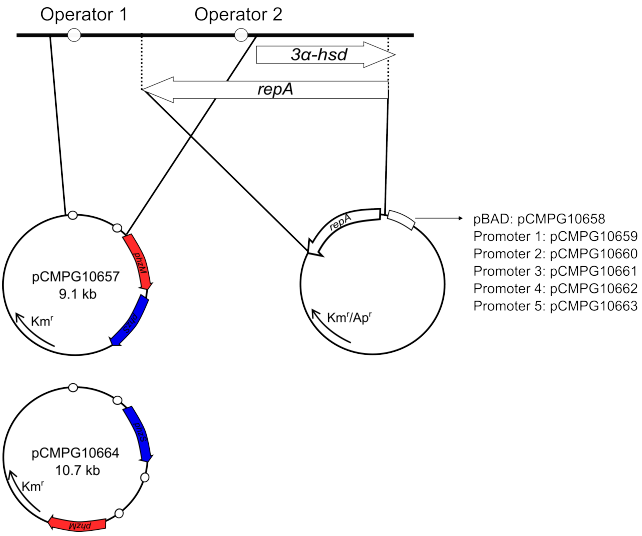


Figure 4.12: Design of plasmids pCMPG10657 and pCMPG10664. The *phzM* and *phzS* genes of pUCP-MS were copied and placed in plasmid pBBR1MCS-2. The two operator sites of *C. testosteroni* were copied and placed upstream of the *phzM* gene to result in plasmid pCMPG10657. For plasmid pCMPG10664 the two operator sites were copied once more and placed upstream of the *phzS* gene.

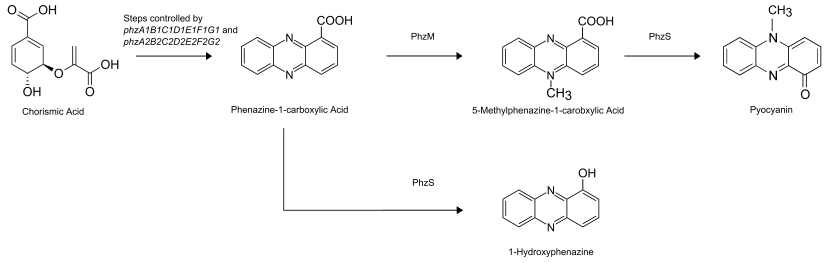


Figure 4.13: Detailed scheme of the pyocyanin pathway of *P. aeruginosa*. Chorismic acid is converted to PCA by the gene products of the two homologous operons *phzA1B1C1D1E1F1G1* and *phzA2B2C2D2E2F2G2*. PCA is converted to 5-MCA by PhzM from the *phzM* gene. 5-MCA is converted to pyocyanin by PhzS, product of the *phzS* gene. PhzS can also convert PCA to 1-OH-PHZ.

4.4 Discussion

Table 4.3: List of plasmids needed for the construction of bioreporters described in this chapter.

Plasmid	Function
pCMPG10658	Arabinose inducible RepA
pCMPG10659	Constitutive RepA (Strong1RepA)
pCMPG10660	Constitutive RepA (Weak1RepA)
pCMPG10661	Constitutive RepA (Weak2RepA)
pCMPG10662	Constitutive RepA (Strong2RepA)
pCMPG10663	Constitutive RepA (MediumRepA)
pCMPG10654	Production of GFP in the presence of steroid hormones
pCMPG10602	Production of enzymes that convert chorismic acid into PCA
pCMPG10657	Production of pyocyanin in the presence of steroid hormones
pCMPG10664	Production of pyocyanin in the presence of steroid hormones

In this chapter the steroid hormone responsive protein RepA and the two operator sites it binds to were used as an input module and combined with output modules GFP and pyocyanin, in an effort to create two whole-cell bacterial steroid hormone bioreporters. The GFP-producing steroid hormone bioreporter is able to detect the presence of testosterone starting from 250 μM, 17β-estradiol, 17α-ethinylestradiol, estrone and progesterone from 1 mM. We were unable to construct a functional pyocyanin-producing steroid hormone bioreporter.

The GFP expression of bacteria transformed with plasmids pCMPG10654 and one of pCMPG10658-pCMPG10663 (table 4.3) is repressed in the absence of steroid hormones. The separation of the two operator sites and repressor RepA into two different plasmids was therefore succesful. However, high concentrations of steroid hormones ($\geqslant$ 250 μM) affect both absorbance and fluorescence measurements. A correction factor was determined experimentally and applied to the results. It should be noted that steroid hormones are not detected in such high concentrations in the environment, measurements of environmental samples would therefore not be affected and a correction factor would not be needed. However, because the absorbance was reduced and the fluorescence intensity increased by the correction factor, the results of the measurements are evidently artificially increased. The corrected results are exaggerated and skewed to what is expected and should therefore only be regarded as an indication. Steroid hormones do not influence the pyocyanin-producing bioreporter in the same way because there is no need for fluorescence or absorbance measurements. For this reason, the results of the GFP-producing bioreporter were to be used as a preliminary screen for the most optimal

promoter/RBS combination, after which the system would be finetuned for both the fluorescent and the pyocyanin-producing bioreporter. The results indicate that the fluorescent bioreporter is able to detect the presence of all measured steroid hormones in a dose-response manner. The most optimal combination of plasmids is that of plasmids pCMPG10654 and pCMPG10662 (Strong2RepA). Strong2RepA appears to yield the best results, both in dose-responseness and amount of fluorescence produced. Weak2RepA (pCMPG10661) also displays a decent dose-response relationship but the amount of measurable fluorescence is significantly lower. Both are in stark contrast to Strong1RepA (pCMPG10659) which displays both low dose-responseness and low measured fluorescence. Nonetheless, both plasmid pCMPG10662 and pCMPG10659 contain a strong constitutive promoter/RBS combination while plasmid pCMPG10661 contains a weak constitutive promoter/RBS combination. Plasmids pCMPG10659 and pCMPG10660 share RBS B0034, which is a high efficiency RBS [74], and differ only in promoters, whereby the promoter of pCMPG10659 (J23100) has higher constitutive activity than the promoter of pCMPG10660 (J23114) [73]. Plasmid pCMPG10662 is made up of promoter J23110 (medium activity) and RBS B0031 (weak efficiency), this results in a strong constitutive promoter/RBS combination. The specific combination of J23114/B0034 and J23110/B0031 appear to be the best suited combinations to regulate the expression of repressor RepA. All plasmids were retained for future use with the pyocyanin-producing bioreporter as well.

The GFP-producing bioreporter uses the same components as COSS but the results are vastly different since COSS is significantly faster and more sensitive. The most important probable cause of this difference in result is the fact that COSS uses *C. testosteroni* as its chassis while the bioreporter described in this chapter incorporated *C. testosteroni* genes into *E. coli*. While RepA is the main regulator of the 3α-HSD/CR gene, other regulators are active in *C. testosteroni* such as RepB that binds to the transcribed 3α-HSD/CR mRNA [64, 220, 221]. It was theorized that RepB would not bind the GFP mRNA and would therefore not be helpful in the regulation of the proposed steroid hormone bioreporter. Nevertheless RepB might have other effects on the expression of 3α-HSD/CR. Likewise, regulator TeiR is a membrane bound kinase that enhances the expression of steroid catabolic genes [64]. Literature states that the presence of TeiR did not have a significant effect on the expression of 3α-HSD/CR [64]. This is proven by the sensitivity of the cell-free system where membrane bound TeiR cannot exert any effects. Yet, all these regulators are present in COSS, which displays better detection properties, so they might have a significant effect on the expression of GFP after all. More research is needed to identify the essential regulators of 3α-HSD/CR.
Another probable cause of the superior sensitivity of COSS might be that the *gfp* gene is incorporated into the chromosome of *C. testosteroni* instead

of using plasmids. Not having to use multiple plasmids decreases the risk of the metabolic load having an influence on the growth and survival of the culture and therefore on the detection of the steroid hormones [62]. In addition, the separation of the operator sites and the RepA protein is less efficient as compared to COSS where all concerned genes are in close proximity.
Lastly, even though steroid hormones are hydrophobic molecules that can freely pass cellular membranes by means of diffusion [140], the cellular membrane is still an obstacle that needs to be passed before a signal can be produced. The cell-free COSS system bypasses the cellular membrane by removing it from the setup. After the construction of a more robust *E. coli*-based bioreporter it would be interesting to research a cell-free setup to improve on sensitivity.

Bacteria transformed with plasmid pCMPG10657 or pCMPG10664 (table 4.3) were unable to produce pyocyanin, even in the absence of the repressor RepA. As mentioned in chapter 3, plasmid pUCP-MS was constructed to study the production of pyocyanin. The design of the plasmid might therefore not be suitable for use as a reporter system. A balance has to be maintained between the expression of both genes in order to produce pyocyanin. Plasmid pCMPG10657 was designed much like pUCP-MS, even though the two operator sites seem to be more complicated than P_{BAD} or P_{BC} from the auxin bioreporter and therefore unable to maintain the balance between the expression of the *phzM* and the *phzS* gene in the current design.
pCMPG10664 is different in design because the two operator sites, together with its promoter and RBS, was placed upstream of both gene *phzM* and gene *phzS*. Since plasmid pCMPG10664 also fails to produce pyocyanin, a more suitable explanation is needed.

One other possible explanation is that pyocyanin is a virulence factor and as such displays antibiotic behaviour against bacteria, nematodes, fungi and plants by causing the formation of reactive oxygen species [68, 104]. *P. aeruginosa* not only adapted to withstand the toxicity of pyocyanin [12], studies have demonstrated that phenazines, and pyocyanin in particular, are beneficial for the competitiveness and survival of its producers [39, 123]. *E. coli* bacteria on the other hand are greatly affected by pyocyanin, their cell growth is decreased by the accumulation of reactive oxygen species. Although the pyocyanin-producing genes are highly conserved in *P. aeruginosa* [123], *E. coli* will not conserve these genes since its wild type does not contain the pyocyanin-producing genes and *E. coli* has not got any evolutionary advantage in producing pyocyanin. Perhaps an initial expression of pyocyanin exerts a evolutionary pressure on the *E. coli* bacteria resulting in a loss of function mutation in either the *phzM* or *phzS* gene. Such an evolutionary pressure, from the toxicity of pyocyanin, appears to be absent in the pyocyanin-producing auxin bioreporter at first glance. But there too, signs of accumulation of a phenazine, presumably 5-MCA, are present

(Fig. 3.19–A and 3.18–A oxidation peak of -50mV). So either gene *phzS* is susceptible to evolutionary change under pressure of virulence factor pyocyanin or the expression is in some way hindered in the proposed design.
Another source of pressure on the growth of both the steroid hormone bioreporters is the use of multiple plasmids [62]. Efforts were made to place the two operator sites and the *repA* gene on one plasmid, but this has proven to be very difficult due to the high similarity in DNA sequence of the operator sites and the *repA* gene. The efforts were abandoned in favour of a multiple plasmid system which probably has a detrimental effect on the function of the bioreporters. Further research is needed to elucidate this.

In summary, the steroid hormone bioreporter's production of two different reporter systems (GFP and pyocyanin) was measured in response to five different steroid hormones (testosterone, 17β-estradiol, 17α-ethinylestradiol, estrone and progesterone).
The GFP-producing steroid hormone bioreporter, pCMPG10654&pCMPG10658-pCMPG10663/TOP10, can detect all tested steroid hormones in a concentration range of 250 μM–1 mM. However, a correction factor was applied to these results.
The construction of the pyocyanin-producing steroid hormone bioreporter was unsuccesful as no construct was able to produce pyocyanin in response to steroid hormones but the elements provided here can lead to the construction of an electrochemical steroid hormone biosensor in future research.

Chapter 5

Conclusions and Perspectives

5.1 Comparison of the constructed whole-cell bacterial bioreporters with traditional detection methods

The previous two chapters described the design and construction of four whole-cell bacterial hormone bioreporters, two auxins bioreporters and two steroid hormone bioreporters, followed by the results obtained with these bioreporters. In this chapter, the use of whole-cell bacterial bioreporters is compared to traditional detection methods for hormones as described in section 1.5. Comparisons will be made in four categories: the **speed** with which the hormones are detected, the **convenience** of the method and lastly the **sensitivity** and **specificity** of the method. An overview of the comparison is shown in table 5.1.

The time required to make measurements using the whole-cell bacterial bioreporters is relatively short. First, an overnight culture of the appropriate bioreporter is prepared. Next, the unknown quantity of hormones is added to this culture and is left to incubate for 4–8 hours for bacteria containing pCMPG10652 (auxins) and 24 hours for bacteria carrying pCMPG10654 (steroid hormones). Results of measurements can be expected in 16–36 hours after sample preparation has started. Traditional methods like MS/GC/LC and immunoreporters can detect accurate concentrations in a manner of hours [5, 78, 107, 109, 138, 211, 230], whereas methods that determine concentrations based on biological effects require days to weeks for an estimate of estrogenicity

[26, 57, 58, 145, 148, 164].

To measure an unkown concentration of hormones, whole-cell bacterial bioreporters require little preparation and training. An overnight culture of the appropriate bioreporter needs to be prepared. This culture needs to be distributed to a microtiterplate for fluorescent measurements, or test tubes for electrochemical measurements. Afterwards the unknown quantity of hormones is added and left to incubate for 4 to 24 hours. The fluorescence measurements are made using a microtiter plate reader, the electrochemical measurements using the MEA and an autolab. Both are very easily taught and performed. This in stark contrast to the methods that measure the concentration directly, like MS/GC/LC or immunosensors [5, 80, 40, 78, 107, 110, 138, 230]. While the preparation time for these experiments is shorter, they require extensive training and are far more complex than the preparations required for whole-cell bacterial bioreporters. Furthermore, the cost of immunosensors is rather high [107, 138, 230]. The E-screen relies on the use of a breast cancer cell line which requires laborious upkeep and intensive training [145, 181, 182]. For vitellogenin assays live fish need to be transported to specific places in surface water after which blood is collected through operation and chromatography. Finally, the blood is analysed using ELISA [36, 57, 144, 148]. YES and YAS are relatively labour-intensive because they make use of the *lacZ* reporter system [58, 164]. The DII-VENUS sensor plants are transplanted to a different medium after five days and scanned every two to five minutes for two hours using a laser-scanning confocal microscope followed by more elaborate tissue-specific kinetics [26].
An important addition to both speed and convenience of whole-cell bacterial bioreporters needs to be made. While bioreporters are indeed fast and easy to use, the construction of bacterial bioreporters requires a big initial investment of both time and effort.

The GFP-producing auxin bioreporter, described in this work, is able to detect 4-HPA and PAA concentrations between 2 mg/L and 450 mg/L. The pyocyanin-producing auxin bioreporter is able to detect 4-HPA concentrations between 289 μg/L and 2 mg/L and PAA concentrations between 2 mg/L and 17 mg/L. The GFP-producing steroid hormone bioreporter is able to detect a variety of steroid hormones in concentrations between 72 mg/L and 865 mg/L. The pyocyanin-producing steroid hormone bioreporter is not able to detect any steroid hormones. Table 1.1 at page 11 lists the steroid hormone concentrations found in Flemish water bodies, these results show that the sensitivity of the steroid hormone bioreporter is insufficient to detect environmental relevant concentrations with respect to pollution. A bioreporter should preferably be able to detect concentrations within the range of the inferred PNEC values. Giridhar *et al.* demonstrated that externally added PAA concentrations between 0.5 mg/L and 2 mg/L are relevant concentrations for plant growth [38, 61], other

studies report concentrations as high as 13 mg/L [234]. Both auxin bioassays are therefore able to detect environmentally relevant concentrations. However, MS/GC/LC and immunoreporters can detect concentrations as low as 10 pg/L [78, 109, 211] and 50 ng/L respectively [66, 230]. The E-screen and YES/YAS detect E2 concentrations as low as 3-8 ng/L [145, 164]. DII-VENUS has a detection limit of 175 ng/L [26]. Both the steroid hormone bioreporter and the auxin bioreporter are therefore low in sensitivity, especially compared to the MS/GC/LC. This is currently the biggest disadvantage of this method, for which some possible solutions are listed in section 5.4.
The auxin bioreporter is specific to 4-HPA and PAA, two compounds with a similar structure.
For the steroid hormone bioreporter, further tests need to be performed to more existensively determine the specificity of the reporter in response to other EDC. MS/GC/LC and immunosensors can be made highly specific to steroid hormones and auxins [5, 80, 40, 78, 107, 110, 138, 230]. Vitellogenin assays, E-screen and YES/YAS are not specific to steroid hormones, they react to all estrogenic compounds [36, 57, 58, 144, 145, 148, 164, 181, 182]. DII-VENUS is very specific to auxin IAA [26].

The constructed bioreporters described in this work can compete with existing methods in both speed and convenience but lack severely in sensitivity. The specificity of the bioreporters is still largely unknown.

5.1.1 Comparative table

Table 5.1: Table comparing the properties of each method.

Method	**Preparation time**	**Measurement time**	**Sensitivity**	**Specificity**
GC/LC-MS	1–2 h	1–2 h	10 pg/L	specific molecules
Immunoassays	>3 h	30 min	50 ng/L	specific molecules
Vitellogenin assay	1 day	1–3 weeks	ng/L	estrogenic compounds
E-screen	1 day	6 days	8 ng/L	estrogenic compounds
YES/YAS	1 day	1–3 days	3–8 ng/L	estrogenic compounds
COSS (cell-based)	12 h	30 min	1.6 μg/L	steroids
COSS (cell-free)	14 h	30 min	29 pg/L	steroids
DII-VENUS	5 days	2 h	175 ng/L	IAA
Bacterial bioreporters	12 h	4–24 h	2 mg/L	

5.2 Comparison of the constructed whole-cell bacterial bioreporters with existing whole-cell bacterial bioreporters and biosensors for organic compounds

A large number of bioreporters and biosensors for the detection of organic compounds already exists. These are relatively simple reporters that carry a constitutively expressed responsive protein and a promoter which is regulated by this protein followed by a reporter gene. In nearly all cases the bioreporter relies on an optical reporter system [95, 174, 218]. They are therefore similar in design to the fluorescent auxin bioreporter but different from the electrochemical auxin biosensor and the steroid hormone bioreporter. The pyocyanin reporter system is a very promising system since it can easily be integrated with a MEA or other electrode setup to construct a biosensor. While fluorescent or bioluminent bioreporters are often integrated with a fluorometer or luminometer, resulting in a biosensor [22, 196, 207], the electrochemical biosensor is interesting because it immediately transduces the signal of detection to an electrical system without needing an intermediate signal (*e.g. GFP or luciferase*). This allows for the construction of more compact biosensors, all it needs is a MEA that is adapted to be used with pyocyanin-producing bacteria.
The production of pyocyanin itself on the other hand is a lot more complex than that of GFP or luciferase. The pyocyanin reporter system consists of two operons of genes needed to produce pyocyanin instead of a single gene as for GFP and luciferase. This requires a larger effort from the bacterial cell, moreover, since all these intermediate proteins need to expressed, a lot more time is needed as well.

The detection of organic compounds by the auxin bioreporters is similar to that of existing bioreporters whereby a single transcriptional regulator binds the analyte and activates the expression of the reporter genes. Although the steroid hormone bioreporter also consists of a single transcriptional regulator, the regulation of the expression of the reporter genes is more complex because the regulator gene overlaps with the gene it regulates. It has proven impossible to place the regulator gene and the operator sites to which it binds in one plasmid due to the high similarity in DNA sequence of the operator sites and the regulator gene. This makes the steroid hormone detection system unintentionally more complex than that of existing bioreporters. Additionally, the need for multiple plasmids places a heavier load on the bacterial cells as well.

In general, existing bioreporters and biosensors for organic compounds possess a

detection sensitivity between 1 nM and 40 μM [205]. The bioreporters described in this work are less sensitive and are constricted to concentrations above 2 μM for the auxin electrochemical biosensor, 30 μM for the auxin fluorescent bioreporter and 250 μM for the steroid hormone bioreporter. This might be caused by the higher complexity of both the detection and reporter systems of these bioreporters. Literature on existing bioreporters is silent on how the analytes pass the cellular membrane, this makes it hard to determine whether existing bioreporters rely on transporter proteins or not. However, as mentioned in chapter 3, incorporating an auxin transporter in the design of the auxin bioreporter could increase its sensitivity to levels rivaling that of existing bioreporters. Additional research on the effects on transporter proteins on detection by bioreporters would therefore be useful for the entire field.

5.3 Should we use genetically modified organisms as bioreporters/biosensors?

Since the conception of genetical engineering it has only become easier to design and construct organisms adapted to certain environments or situations. Synthetic biology opens the way to engineer living systems to improve our quality of life, but it also creates the opportunity to engineer living systems that can cause harm to human beings or the environment in general. We can expect huge benefits from synthetic biology but there are risks as well. To address these issues, several bodies and organizations have started discussions to try and minimize the risks associated with synthetic biology [172]. This is an on-going discussion and it is important to think in advance about all the open questions in order to inform the public. Some of these open questions are addressed in this section.

The most pertinent question regarding genetically engineered organisms is whether or not these organisms should be used in situations were the risk exists that genetically engineered organsims get introduced into the environment. Several inherent risks are associated with this issue [180], including the creation of new, or stronger pathogens, disruption of biotic communities and irreversible loss in species diversity. While it is believed that with careful design and proper planning genetically engineered organisms can be introduced with minimal ecological risk [180], it is still impossible to foresee all outcomes of such an introduction. Pleoiotropic effects (secondary phenotypic effects of a single gene mutation) for example might be easily overlooked. Even though the genetic alterations produced now are more precise and directed than those produced using selection and other strategies, this does not ensure that unintended changes

in the genome have been prevented or that all ecologically important aspects of the phenotype can be predicted for the environment in which the organism will be introduced. On the other hand, many engineered organisms will probably be less fit than the original organism [194] due to an unbalanced metabolic load or other disruptions inside the microbial cell. Genetically modified organisms introduced in the environment might therefore disappear very fast. Either way, the bioreporters constructed and described in this work are not going to be introduced to the environment in the near future.
There are still a lot of unknowns in the debate of the use of genetically modified organisms for bioremediation. These unknowns should be further investigated so future risks can be reduced, preferably within the context of a scientifically based regulatory policy [194]. At the same time it might be beneficial to inform the public of these concepts in a relatable context to reduce the lingering fear asociated with the use of genetically modified organisms.
One important question remains. While we possess the knowledge and technology to create genetically modified organisms, is it ethical to do so? Should mankind "toy with nature"? No one can really say for sure but I believe that genetically engineered organisms can prove to be a huge contribution to the future of mankind.

5.4 Future perspectives

Both the auxin and the steroid hormone bioreporter are able to detect their respective analytes to a certain degree. Yet, there is still room for improvement of both bioreporters, in particular to the limit of detection which is rather high for the constructed bioreporters and should be decreased. In this section some suggestions for improvement of the detection systems and the reporter systems are offered as future perspectives.

The auxin detection system relies on auxin responsive protein HpaA that binds 4-HPA and PAA. Probably the biggest limitation of the constructed auxin bioreporters is the transport over the cellular membrane. While literature and experiments show that 4-HPA and PAA are able to pass the cellular membrane, the limits of detection can probably be decreased by adding transporter protein HpaX to the bioreporter. HpaX would actively transport 4-HPA in the cell and reduce the time needed for a dose-response reporter molecule production and reduce the detection limits [156]. For the fluorescent auxin bioreporter the detection range is 31.25 μM to 3 mM. Prieto and García report a detection range of 100 nM to 1 μM when transporter protein HpaA is expressed as well. Introducing HpaX to the fluorescent auxin bioreporter might therefore lower the detection range to 100 nM–1 μM. This means that concentrations between

1 μM and 31.25 μM would not be detected. And since auxins are mostly active in concentrations between 0.1 and 10 μM [114], this would be a disadvantage. A bioreporter containing 4-HPA transporter protein HpaX should be constructed and characterized to verify if this is the case. The use of a transporter protein would only work for 4-HPA since no transporter proteins have been identified yet for PAA or IAA [132, 183].
Additionally, a cell-free setup of the constructed bioreporters should be looked into, similar to COSS [121]. Bacterial membranes form a barrier for the detection of analytes, removing them should increase the sensitivity of the bioreporter.
With the recent identification of the *iac* genes, coding for enzymes that act in the degradation of IAA [170], a bioreporter can possibly be constructed that detects concentrations of IAA and possible other auxins with a similar structure such as IBA and 4-Cl-IAA.

The steroid hormone input module uses steroid responsive protein RepA and two operator sites to which RepA binds. A lot of time was lost in the construction of this bioreporter because of the complexity of the input module. Firstly, the two operator sites had to be spliced to the *gfp* gene to be able to express, even one extra base between the second operator and the start codon of the downstream gene made transcription impossible. Secondly, the high similarity in DNA sequence of the operator sites and the *repA* gene caused multiple problems that made it impossible to place the operator sites and the *repA* gene in one plasmid. The latter can possibly be solved by buying a synthesized gene fragment containing the DNA sequence of the operator sites and the *repA* gene. This would reduce the number of plasmids needed, and therefore the metabolic load on the bacterial cell.
Even though literature suggests that no regulators other than RepA are needed for the function of the bioreporter [64, 220, 221], it might be possible that some other regulator exists that explains the discrepancy in the results between COSS and the bioreporters described here. A series of experiments on the expression of GFP in the presence of all known regulators might identify a regulator that improves the detection of steroid hormones.
Lastly, here too, a cell-free setup of the constructed bioreporters should be looked into.

The GFP reporter system comprises the *gfp* gene. The induced cultures of GFP-producing bioreporters is cultured at 37°C in black 96-well plates with clear bottoms. At these temperatures a part of the bacteria's medium evaporates which influences the results of the measurements significantly. By removing the need for 96-well plates the reproducibility of the measurements will be increased, as well as the reliability of the results. At the same time it would be interesting to integrate the GFP-producing bioreporters with a detector and construct an integrated fluorescent biosensor. This might possibly be achieved by using an

optical fibre bundle as described in figure 1.3 [207].

The pyocyanin reporter system consists of two operons, one containing the *phzA2B2C2D2E2F2G2* genes, needed to convert chorismic acid to PCA, under control of the P_{BAD} promoter. The other operon contains genes *phzM* and *phzS* that convert PCA to pyocyanin.
Since the response of the arabinose–P_{BAD} system in individual cells to arabinose concentrations is not linear [89, 176], it might be interesting to replace the P_{BAD} promoter with a constitutive promoter or the promoter of the *lac* operon to ensure a more linear production of PCA. The promoter of the *lac* operon is induced by the synthetic isopropyl-β-D-thiogalactoside (IPTG). IPTG can freely pass the cellular membrane, in contrast to arabinose which requires active transporters, and cannot be metabolized by the cell, also unlike arabinose. This would increase the reproducibility of the bioreporter. Another solution is just adding a specific concentration of PCA to the medium. Since PCA is a small diffusible molecule that easily passes the cellular membrane [151] it is not necessary to produce PCA internally. This would also reduce the number of plasmids needed and therefore the metabolic load on the bacterial cell.
The additional peak of oxidation at -50 mV observed in figures 3.18–A, 3.19–A and 3.20–A cannot be attributed to the presence of pyocyanin. The height of the additional peak is inversely related to the concentration of auxin whereby low concentrations show a higher peak at -50 mV. Therefore, another redox-active molecule is being produced in the presence of low amounts of auxins, even in the presence of IAA, which does not induce expression of pyocyanin itself. This molecule is likely also a phenazine or a precursor since it oxidizes at a negative voltage, but could not be identified yet. There are a number of possible candidates for this phenazine since the conversion of chorismic acid to pyocyanin has many intermediates. The most likely candidate is PCA because this intermediate is produced continuously by the products of plasmid pCMPG10602 while the expression of *phzM* and *phzS*, which would convert PCA, at lower concentrations of auxins will be low. Thus the amount of PCA will build up because it cannot be converted to pyocyanin. Nevertheless, the oxidation of PCA happens at -400 mV [17] and not at -50 mV. The only intermediate which oxidizes at approximately -50 mV is 5-MCA whose oxidation happens at -70 mV and reduction at -100 mV [17]. 5-MCA is thus very likely the intermediate phenazine that is visible on the voltammograms. 5-MCA is converted from PCA by PhzM and is normally converted to pyocyanin by PhzS. This might suggest that the expression of *phzS* is lower than that of *phzM* at low concentrations of auxins. Here too, further research is needed to determine the source of the accumulating molecule.
In the genome of *P. aeruginosa*, *phzM* and *phzS* are located on opposite ends of operon *phzA1B1C1D1E1F1G1*, both with their own promoter. In pUCP-MS, both genes were placed under control of one P_{lac} promoter. pUCP-MS

was originally constructed to study the production of pyocyanin and might therefore not be suitable to use as a reporter system. An important future adaptation to look into is a more efficient application of the *phzM* and *phzS* genes whereby both are transcribed in equal fashion. This should reduce the possibility of accumulation of intermediates and lead to a more efficient production of pyocyanin.
Another solution to reduce the negative effect pyocyanin has on the growth and survival of *E. coli* cells is to use the more pyocyanin resistant *E. coli* strain [208], specifically created to perform pyocyanin-producing experiments with E. coli.
Finally the pyocyanin biosensor can be improved as well. The MEA used in all the experiments is a generic MEA with a bulky setup. The design of a specific MEA, adapted to be used with the pyocyanin-producing bioreporter, could improve the reproducibility and sensitivity of the biosensor. Furthermore, The MEA might possibly be adapted in such a way to realize *in situ* applications of a steroid hormone biosensor.

Although the bioreporters constructed in this work are able to dose-responsely detect auxins and steroid sex hormones, the future perspectives listed above show that there is still much room for improvement. The constructs presented here can therefore serve as preliminary bioreporters that can be improved in the future for the construction of efficient, sensitive hormone bioreporters.

Bibliography

[1] Abd-El-Haleem, D., Ripp, S., Scott, C., and Sayler, G. S. A *luxCDABE*-based bioluminescent bioreporter for the detection of phenol. *Journal of Industrial Microbiology and Biotechnology 29* (2002), 233–237.

[2] Abraham, J. M., and Simon, G. L. *Comamonas testosteroni* bacteremia: *A Case Report and Review of the Literature. Infectious Diseases in Clinical Practice 15* (2007), 272–273.

[3] Albert, D., Jonik, R., and Walsh, M. Hormone-dependent aggression in male and female rats: Experiential, hormonal, and neural foundations. *Neuroscience and Biobehavioral Reviews 16* (1992), 177–192.

[4] Ames, B. N., Durston, W. E., Yamasaki, E., and Lee, F. D. Carcinogens are mutagens: A simple test system combining liver homogenates for activation and bacteria for detection. *Proceedings of the National Academy of Sciences of the USA 70* (1973), 2281–2285.

[5] Andrási, N., Molnár, B., Dobos, B., Vasanits-Zsigrai, A., Záray, G., and Molnár-Perl, I. Determination of steroids in the dissolved and in the suspended phases of wastewater and Danube river samples by gas chromatography, tandem mass spectrometry. *Talanta 115* (2013), 367–373.

[6] Applegate, B., Kelly, C., Lackey, L., McPherson, J., Kehrmeyer, S., Menn, F.-M., Bienkowski, P., and Sayler, G. *Pseudomonas putida* b2: a *tod-lux* bioluminescent reporter for toluene and trichloroethylene co-metabolism. *Journal of Industrial Microbiology and Biotechnology 18* (1997), 4–9.

[7] Aquiris. *Sewage treatment plant Brussels.* `http://www.aquiris.be/en/history.php`.

[8] Archer, J. The influence of testosterone on human aggression. *British Journal of Psychology 82* (1991), 1–28.

[9] Ashrafi, M., Gosili, R., Hosseini, R., Arabipoor, A., Ahmadi, J., and Chehrazi, M. Risk of gestational diabetes mellitus in patients undergoing assisted reproductive techniques. *European Journal of Obstetrics & Gynecology and Reproductive Biology 176* (2014), 149–152.

[10] Azziz, R., Carmina, E., Dewailly, D., Diamanti-Kandarakis, E., Escobar-Morreale, H., Futterweit, W., Janssen, O. E., Legro, R. S., Norman, R. J., Taylor, A. E., and Witchel, S. F. The androgen excess and PCOS society criteria for the polycystic ovary syndrome: the complete task force report. *Fertility and Sterility 91* (2009), 459–488.

[11] Bagatelli, C. J., and Bremner, W. J. Androgens in men — uses and abuses. *Drug Therapy 334* (1996), 707–714.

[12] Baron, S. S., and Rowe, J. J. Antibiotic action of pyocyanin. *Antimicrobial Agents and Chemotherapy 20* (1981), 814–820.

[13] Bartelt-Hunt, S., Snow, D. D., Damon-Powell, T., and Miesbach, D. Occurrence of steroid hormones and antibiotics in shallow groundwater impacted by livestock waste control facilities. *Journal of Contaminant Hydrology 123* (2011), 94–103.

[14] Barzen, C., Brecht, A., and Gauglitz, G. Optical multiple-analyte immunosensor for water pollution control. *Biosensors and Bioelectronics 17* (2002), 289–295.

[15] Baumann, B., and van der Meer, J. R. Analysis of bioavailable arsenic in rice with whole cell living bioreporter bacteria. *Journal of Agricultural and Food Chemistry 55* (2007), 2115–2120.

[16] Baumann, L., Knörr, S., Keiter, S., Rehberger, K., Volz, S., Schiller, V., Fenske, M., Holbech, H., Segner, H., and Braunbeck, T. Reversibility of endocrine disruption in zebrafish (*Danio rerio*) after discontinued exposure to the estrogen 17α-ethinylestradiol. *Toxicology and Applied Pharmacology 278* (2014), 230–237.

[17] Bellin, D. L., Sakhtah, H., Rosenstein, J. K., Levine, P. M., Thimot, J., Emmett, K., Dietrich, L. E., and Shepard, K. L. Integrated circuit-based electrochemical sensor for spatially resolved detection of redox-active metabolites in biofilms. *Nature Communications 5* (2014), 1–10.

[18] Bergman, A., Heindel, J. J., Jobling, S., Kidd, K. A., and Zoeller, R. T., Eds. *State of the Science of Endocrine Disrupting Chemicals - 2012*. United Nations Environment Programme and the World Health Organization, 2013.

[19] Bhandari, R. K., Deem, S. L., Holliday, D. K., Jandegian, C. M., Kassotis, C. D., Nagel, S. C., Tillitt, D. E., vom Saal, F. S., and Rosenfeld, C. S. Effects of the environmental estrogenic contaminants bisphenol A and 17α-ethinyl estradiol on sexual development and adult behaviors in aquatic wildlife species. *General and Comparative Endocrinology* (2014).

[20] Bhat, H. K., Calaf, G., Hei, T. K., Loya, T., and Vadgama, J. V. Critical role of oxidative stress in estrogen-induced carcinogenesis. *Proceedings of the National Academy of Sciences 100* (2003), 3913–3918.

[21] Bijlsma, J. J., and Groisman, E. A. The PhoP/PhoQ system controls the intramacrophage type three secretion system of *Salmonella enterica*. *Molecular Microbiology 57* (2005), 85–96.

[22] Bolton, E. K., Sayler, G. S., Nivens, D. E., Rochelle, J. M., Ripp, S., and Simpson, M. L. Iintegrated CMOS photodetectors and signal processing for very low-level chemical sensing with the bioluminescent bioreporter integrated circuit. *Sensors and Actuators B 85* (2002), 179–185.

[23] Boon, N., Goris, J., Vos, P. D., Verstraete, W., and Top, E. M. Bioaugmentation of activated sludge by an indigenous 3-chloroaniline-degrading *Comamonas testosteroni* strain, I2gfp. *Applied and Environmental Microbiology 66* (2000), 2906–2913.

[24] Boruah, H. P. D., Chauhan, P. S., jong Yim, W., Han, G.-H., and Sa, T.-M. Comparison of plant growth promoting *Methylobacterium* spp. and exogenous indole-3-acetic acid application on red peppers and tomato seedling development. *Korean Journal of Soil Science and Fertilizer 43* (2010), 96–104.

[25] Brown, N. J. Aldosterone and vascular inflammation. *Hypertension 51* (2008), 161–167.

[26] Brunoud, G., Wells, D. M., Oliva, M., Larrieu, A., Burrow, V. M. A. H., Beeckman, T., Kepinski, S., Traas, J., Bennett, M. J., and Vernoux, T. A novel sensor to map auxin respose and distribution at high spatio-temporal resolution. *Nature 482* (2012), 103–108.

[27] Caldwell, D. J., Mastrocco, F., Anderson, P. D., Länge, R., and Sumpter, J. P. Predicted-no-effect concentrations for the steroid estrogens estrone, 17β-estradiol, estriol and 17α-ethinylestradiol. *Environmental Toxicology and Chemistry 31* (2012), 1396–1406.

[28] Casadaban, M. J. Transposition and fusion of the *lac* genes to selected promoters in *Escherichia coli* using bacteriophage lambda and mu. *Journal of Molecular Biology 104* (1976), 541–555.

[29] Close, D. M., Ripp, S., and Sayler, G. S. Reporter proteins in whole-cell optical bioreporter detection systems, biosensor integrations, and biosensing applications. *Sensors 9* (2009), 9147–9174.

[30] Cooper, G. R., Staples, E. D., Iczkowski, K. A., and Clancy, C. J. *Comamonas (Pseudomonas) testosteroni* endocarditis. *Cardiovascular Pathology 14* (2005), 145–149.

[31] Cooper, L. A., and Page, S. T. Androgens and prostate disease. *Asian Journal of Andrology 16* (2014), 248–255.

[32] Corbisier, P., Ji, G., Nuyts, G., Mergeay, M., and Silver, S. *luxAB* gene fusions with the arsenic and cadmium resistance operons of *Staphylococcus aureus* plasmid pI258. *FEMS Microbiology Letters 110* (1993), 231–238.

[33] Cormack, B. P., Valdivia, R. H., and Falkow, S. FACS-optimized mutants of the green fluorescent protein (GFP). *Gene 173* (1996), 33–38.

[34] Crews, D., Bull, J. J., and Wibbels, T. Estrogen and sex reversal in turtles: A dose-dependent phenomenon. *General and Comparative Endocrinology 81* (1991), 357–364.

[35] Cronan, J. E. A family of arabinose-inducible *Escherichia coli* expression vectors having pBR322K copy control. *Plasmid 55* (2006), 152–157.

[36] Dammann, A. A., Shappell, N. W., Bartell, S. E., and Schoenfuss, H. L. Comparing biological effects and potencies of estrone and 17β-estradiol in mature fathead minnows, *Pimephales promelas*. *Aquatic Toxicology 105* (2011), 559–568.

[37] Derbyshire, K. M., and Willetts, N. S. Mobilization of the non-conjugative plasmid RSF1010: A genetic analysis of its origin of transfer. *Molecular and General Genetics 206* (1987), 154–160.

[38] Dhaka, N., and Kothari, S. Phenylacetic acid improves bud elongation and *in vitro* plant regeneration efficiency in *Helianthus annuus* L. *Plant Cell Reports 21* (2002), 29–34.

[39] Dietrich, L. E. P., Price-Whelan, A., Petersen, A., Whiteley, M., and Newman, D. K. The phenazine pyocyanin is a terminal signalling factor in the quorum sensing network of *Pseudomonas aeruginosa*. *Molecular Microbiology 61* (2006), 1308–1321.

[40] D'Orazio, G., Asensio-Ramos, M., Hernández-Borges, J., Fanali, S., and Ángel Rodríguez-Delgado, M. Estrogenic compounds determination in water samples by dispersive liquid-liquid microextraction and micellar electrokinetic chromatography coupled to mass spectrometry. *Journal of Chromatography A 1344* (2014), 109–121.

[41] Eguílaz, M., Moreno-Guzmán, M., Campuzano, S., González-Cortés, A., nez Sedeño, P. Y., and Pingarrón, J. M. A electrochemical immunosensor for testosterone using functionalized magnetic beads and screen-printed carbon electrodes. *Biosensors and Bioelectronics 26* (2010), 517–522.

[42] Eisenberger, A., and Westhoff, C. Hormone replacement therapy and venous thromboembolism. *Journal of Steroid Biochemistry & Molecular Biology 142* (2014), 76–82.

[43] El-Gaied, L. F., El-Heba, G. A. A., and El-Sherif, N. A. Effect of growth hormones on some antioxidant parameters and gene expression in tomato. *GM Crops and Food: Biotechnology in Agriculture and the Food Chain 4* (2013), 67–73.

[44] Engström, H. A., Andersson, P. O., and Ohlson, S. A label-free continuous total-internal-reflection-fluorescence-based immunosensor. *Analytical Biochemistry 357* (2006), 159–166.

[45] Esty, D., and Cornelius, P., Eds. *Environmental Performance Measurement: Global Report 2001-2002*. Oxford University Press, New York, 2002.

[46] Esty, D., Levy, M., Kim, C., de Sherbinin, A., Srebotnjak, T., and Mara, V., Eds. *2008 Environmental Performance Index*. New Haven: Yale Center for Environmental Law and Policy, 2008.

[47] European Comission. *Hormones in meat*. `http://ec.europa.eu/food/food/chemicalsafety/contaminants/hormones/index_en.htm`.

[48] European Commission. *WFD Implementation Reports 2012*. `http://ec.europa.eu/environment/water/water-framework/pdf/CWD-2012-379_EN-Vol3_BE.pdf`.

[49] European Commission. *WFD Implementation Reports 2012*. http://ec.europa.eu/environment/water/water-framework/pdf/CWD-2012-379_EN-Vol1.pdf.

[50] European Commission. *WFD Implementation Reports 2012*. http://ec.europa.eu/environment/water/water-framework/pdf/CWD-2012-379_EN-Vol2.pdf.

[51] European Union. *Richtlijn 2008/105/EG van het Europees parlement en de raad*. http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2008:348:0084:0097:nl:PDF.

[52] Fan, Y.-L., Peng, H.-S., Xie, K., and Zhang, X.-H. Measurement and correlation of the solubility of 1-hydroxyphenazine in different solvents at temperature from 278.5 to 333.5 K. *Journal of Shanghai Jiaotong University 18* (2013), 253–256.

[53] Farshad, S., Norouzi, F., Aminshahidi, M., Heidari, B., and Alborzi, A. Two cases of bacteremia due to an unusual pathogen, *Comamonas testosteroni* in iran and a review literature. *The Journal of Infection in Developing Countries 6* (2012), 521–525.

[54] Fayad, P. B., Prévost, M., and Sauvé, S. On-line solid-phase extraction coupled to liquid chromatography tandem mass spectrometry optimized for the analysis of steroid hormones in urban wastewaters. *Talanta 115* (2013), 349–360.

[55] Fenske, M., Maack, G., Schäfers, C., and Segner, H. An environmentally relevant concentration of estrogen induces arrest of male gonad development in zebrafish, *Danio rerio*. *Environmental Toxicology and Chemistry 24* (2005), 1088–1098.

[56] Ferrández, A., García, J. L., and Díaz, E. Transcriptional regulation of the divergent *paa* catabolic operons for phenylacetic acid degradation in *Escherichia coli*. *Journal of Biological Chemistry 276* (2000), 12214–12222.

[57] Flick, R. W., Bencic, D. C., See, M. J., and Biales, A. D. Sensitivity of the vitellogenin assay to diagnose exposure of fathead minnows to 17α-ethynylestradiol. *Aquatic Toxicology 152* (2014), 353–360.

[58] Gaido, K. W., Leonard, L. S., Lovell, S., Gould, J. C., Babai, D., Portier, C. J., and McDonnell, D. P. Evaluation of chemicals with endocrine modulating activity in a yeast-based steroid hormone receptor gene transcription assay. *Toxicology and Applied Pharmacology 143* (1997), 205–212.

[59] Galán, B., Kolb, A., Sanz, J. M., García, J. L., and Prieto, M. A. Molecular determinants of the *hpa* regulatory system of *Escherichia coli*: the HpaR repressor. *Nucleic Acids Research 31* (2003), 6598–6609.

[60] Gann, P. H., Hennekens, C. H., Ma, J., Longcope, C., and Stampfer, M. J. Prospective study of sex hormone levels and risk of prostate cancer. *Journal of the National Cancer Institute 88* (1996), 1118–1126.

[61] Giridhar, P., Ramu, D. V., Reddy, B. O., Rajasekaran, T., and Ravishankar, G. Influence of phenylacetic acid on clonal propagation of *Decalepis hamiltonii* Wight & Arn: an endangered shrub. *In Vitro Cellular & Developmental Biology –Plant 39* (2003), 463–467.

[62] Glick, B. R. Metabolic load and heterologous gene expression. *Biotechnology Advances 13* (1995), 247–261.

[63] Goebel, C., Howe, C. J., Ho, K. K., Nelson, A., Kazlauskas, R., and Trout, G. J. Screening for testosterone abuse in male athletes using the measurement of urinary LH, a revision of the paradigm. *Drug Testing and Analysis 1* (2010), 511–517.

[64] Göhler, A., Xiong, G., Paulsen, S., Trentmann, G., and Maser, E. Testosterone-inducible regulator is a kinase that drives steroid sensing and metabolism in *Comamonas testosteroni*. *Journal of Biological Chemistry 283* (2008), 17380–17390.

[65] Gravel, V., Antoun, H., and Tweddell, R. J. Growth stimulation and fruit yield improvement of greenhouse tomato plants by inoculation with *Pseudomonas putida* or *Trichoderma atroviride*: Possible role of indole acetic acid (IAA). *Soil Biology & Biochemistry 39* (2007), 1968–1977.

[66] Habauzit, D., Chopinau, J., and Roig, B. SPR-based biosensors: a tool for biodetection of hormonal compounds. *Analytical and Bioanalytical Chemistry 387* (2007), 1215–1223.

[67] Harmsen, J. Measuring bioavailability: From a scientific approach to standard methods. *Journal of Environmental Quality 36* (2007), 1420–1428.

[68] Hassan, H. M., and Fridovich, I. Mechanism of the antibiotic action of pyocyanin. *Journal of Bacteriology 141* (1980), 156–163.

[69] Hayes, T. B. Sex determination and primary sex differentiation in amphibians: Genetic and developmental mechanisms. *The Journal of Experimental Zoology 281* (1998), 373–399.

[70] Horton, R. M., hunt, H. D., Ho, S. N., Pullen, J. K., and Pease, L. R. Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. *Gene 77* (1989), 61–68.

[71] Hou, N., Hong, S., Wang, W., Olopade, O. I., Dignam, J. J., and Huo, D. Hormone replacement therapy and breast cancer: Heterogenous risks by race, weight and breast density. *Journal of the National Cancer Institute 105* (2013), 1365–1372.

[72] Husain, S., Jain, A., and Kothari, S. Phenylacetic acid improves bud elongation and *in vitro* plant regeneration efficiency in *Capisicum annuum* l. *Plant Cell Reports 19* (1999), 64–68.

[73] iGEM. *Promoter comparison.* `http://parts.igem.org/Part:BBa_J23100`.

[74] iGEM. *Ribosome Binding Site/Cat page.* `http://parts.igem.org/Ribosome_Binding_Site/Cat_page`.

[75] Iijima, M., Ito, A., Kurosu, S., and Chaki, S. Pharmacological characterization of repeated corticosterone injection-induced depression model in rats. *Brain Research 1359* (2010), 75–80.

[76] International Environmental Law Research Centre. *The right to water in Belgium.* `http://www.ielrc.org/content/f0802.pdf`.

[77] Isidori, M., Cangiano, M., Palermo, F. A., and Parrella, A. E-screen and vitellogenin assay for the detection of the estrogenic activity of alkylphenols and trace elements. *Comparative Biochemistry and Physiology, Part C 152* (2010), 51–56.

[78] Isobe, T., Shiraishi, H., Yasadu, M., Shinoda, A., Suzuki, H., and Morita, M. Determination of estrogens and their conjugates in water using solid-phase extraction followed by liquid chromatography-tandem mass spectrometry. *Journal of Chromatography A 984* (2003), 195–202.

[79] Jacob, C., Jamier, V., and Ba, L. A. Redox-active secondary metabolites. *Current Opinion in Chemical Biology 15* (2011), 149–155.

[80] Janeway, C. A., Travers, P., Walport, M., and Shlomchik, M. J. *Immunobiology*, 6 ed. Garland Science, 2005.

[81] Jethwani, V., and Kothari, S. Phenylacetic acid induced organogenesis in cultured leaf segments of *Dianthus chinensis*. *Plant Cell Reports 15* (1996), 869–872.

[82] Ji, W., Chen, Y., Zhang, H., Zhang, X., Li, Z., and Yu, Y. Cloning, expression and characterization of a putative 7α-hydroxysteroid dehydrogenase in *Comamonas testosteroni*. *Microbiological Research 169* (2014), 148–154.

[83] Jiang, X., Ma, M., Li, J., Lu, A., and Zhong, Z. Bacterial diversity of active sludge in wastewater treatment plant. *Earth Science Frontiers 15* (2008), 163–168.

[84] Jobling, S., Williams, R., Johnson, A., Taylor, A., Gross-Sorokin, M., Nolan, M., Tyler, C. R., van Aerle, R., Santos, E., and Brighty, G. Predicted exposures to steroid estrogens in UK rivers correlate with widespread sexual disruption in wild fish populations. *Environmental Health Perspectives 114* (2006), 32–39.

[85] Johnson, S. A., Fournier, N. M., and Kalynchuk, L. E. Effect of different doses of corticosterone on depression-like behavior and HPA axis responses to a novel stressor. *Behavioural Brain Research 168* (2006), 280–288.

[86] José, M. C. S., Romero, L., and Janeiro, L. V. Effect of indole-3-butyric acid on root formation in *Alnus glutinosa* microcuttings. *Silva Fennica 46* (2012), 643–654.

[87] Karcz, W., and Burdach, Z. A comparison of the effects of IAA and 4-Cl-IAA on growth, proton secretion and membrane potential in maize coleoptile segments. *Journal of Experimental Botany 53* (2002), 1089–1098.

[88] Katayama, M. Synthesis and biological activities of 4-chloroindole-3-acetic acid and its esters. *Bioscience, Biotechnology and Biochemistry 64* (2000), 808–815.

[89] Khlebnikov, A., Risa, Ø., Skaug, T., Carrier, T. A., and Keasling, J. D. Regulatable arabinose-inducible gene expression system with consistent control in all cells of a culture. *Journal of Bacteriology 182* (2000), 7029–7034.

[90] Khlebnikov, A., Skaug, T., and Keasling, J. D. Modulation of gene expression from the arabinose-inducible araBAD promoter. *Journal of Industrial Microbiology & Biotechnology 29* (2002), 34–37.

[91] King, J. M. H., DiGrazia, P. M., Applegate, B., Burlage, R., Sanseverino, J., Dunbar, P., Larimer, F., and Sayler, G. S. Rapid, sensitive bioluminescent reporter technology for naphtalene exposure and biodegradation. *Science 249* (1990), 778–781.

[92] Kissinger, P. T., and Heineman, W. R. Cyclic voltammetry. *Journal of Chemical Education 60* (1983), 702–706.

[93] Kohno, S., Bernhard, M. C., Katsu, Y., Zhu, J., Bryan, T. A., Doheny, B. M., Iguchi, T., and Guillette, L. J. Estrogen receptor 1 (ESR1; ERα), not ESR2 (ERβ), modulates estrogen-induced sex reversal in the american alligator, a species with temperature-dependent sex determination. *Endocrinology* (2015).

[94] Kolodziej, E. P., Harter, T., and Sedlak, D. L. Dairy wastewater, aquaculture, and spawning fish as sources of steroid hormones in the aquatic environment. *Environmental Science & Technology 38* (2004), 6377–6384.

[95] Korpela, M. T., Kurittu, J. S., Karvinen, J. T., and Karp, M. T. A recombinant *Escherichia coli* sensor strain for the detection of tetracyclines. *Analytical Chemistry 70* (1998), 4457–4462.

[96] Kovach, M. E., Elzer, P. H., Hill, D. S., Robertson, G. T., Farris, M. A., II, R. M. R., and Peterson, K. M. Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. *Gene 166* (1995), 175–176.

[97] Kwon, S., and Hermayer, K. Glucocorticoid-induced hyperglycemia. *American Journal of the Medical Sciences 345* (2013), 247–277.

[98] LaCroix, A. Z., Chlebowski, R. T., Manson, J. E., Aragaki, A. K., Johnson, K. C., Martin, L., Margolis, K. L., Stefanick, M. L., Brzyski, R., Curb, J. D., Howard, B. V., Lewis, C. E., and Wactawski-Wende, J. Health outcomes after stopping conjugated equine estrogens among postmenopausal women with prior hysterectomy. *Journal of American Medical Association 305* (2011), 1305–1314.

[99] Lansang, M. C., and Hustak, L. K. Glucocorticoid-induced diabetes and adrenal suppression: How to detect and manage them. *Cleveland Clinic Journal of Medicine 78* (2011), 748–756.

[100] Leveau, J. H. J., and Lindow, S. E. Bioreporters in microbial ecology. *Current Opinion in Microbiology 5* (2002), 259–265.

[101] Lewington, S., Clarke, R., Qizilbash, N., Peto, R., and Collins, R. Age-specific relevance of usual blood pressure to vascular mortality: a meta-analysis of individual data for one million adults in 61 prospectives studies. *Lancet 360* (2002), 1903–1913.

[102] Liang, Y., Zhu, X., Wu, T., Zhao, M., and Liu, H. Rapid and sensitive detection of auxins and flavonoids in plant samples by high-performance liquid chromatography coupled with tandem mass spectrometry. *Journal of Separation Science 35* (2012), 2559–2566.

[103] Liney, K. E., Jobling, S., Shears, J. A., Simpson, P., and Tyler, C. R. Assessing the sensitivity of different life stages for sexual disruption in roach (*Rutilus rutilus*) exposed to effluents from wastewater treatment works. *Environmental Health Perspectives 113* (2005), 1299–1307.

[104] Liu, G. Y., and Nizet, V. Color me bad: microbial pigments as virulence factors. *Trends in Microbiology 17* (2009), 406–413.

[105] Liu, S., Ying, G.-G., Liu, Y.-S., Peng, F.-Q., and He, L.-Y. Degradation of norgestrel by bacteria from activated sludge: Comparison to progesterone. *Environmental Science & Technology 47* (2013), 10266–10276.

[106] Liu, X., Hegeman, A. D., Gardner, G., and Cohen, J. D. Protocol: High-throughput and quantitative assays of auxin and auxin precursors from minute tissue samples. *Plant Methods 8* (2012), 1–17.

[107] liu, X., Wang, X., Zhang, J., Feng, H., Liu, X., and Wong, D. K. Y. Detection of estradiol at an electrochemical immunosensor with a Cu UPD|DTBP-protein G scaffold. *Biosensors and Bioelectronics 35* (2012), 56–62.

[108] Loos, R., Gawlik, B. M., Locoro, G., Rimaviciute, E., Contini, S., and Bidoglio, G. EU-wide survey of polar organic persistent pollutants in european river waters. *Environmental Pollution 157* (2009), 561–568.

[109] López de Alda, M. J., and Barceló, D. Determination of steroid sex hormones and related synthetic compounds considered as endocrine disruptors in water by liquid chromatography-diode array detection-mass spectrometry. *Journal of Chromatography A 892* (2000), 391–406.

[110] López de Alda, M. J., and Barceló, D. Review of analytical methods for the determination of estrogens and progestogens in waste waters. *Fresenius Journal of Analytical Chemistry 371* (2001), 437–447.

[111] Lu, Q., Chen, L., Lu, M., Chen, G., and Zhang, L. Extraction and analysis of auxins in plants using dispersive liquid–liquid microextraction followed by high-performance liquid chromatography with fluorescence detection. *Journal of Agricultural and Food Chemistry 58* (2010), 2763–2770.

[112] Ludwig-Müller, J., Vertocnik, A., and Town, C. D. Analysis of indole-3-butyric acid-induced adventitious root formation on *Arabidopsis* stem segments. *Journal of Experimental Botany 56* (2005), 2095–2105.

[113] Luppa, P. B., Sokoll, L. J., and Chan, D. W. Immunosensors-principles and applications to clinical chemistry. *Clinica Chimica Acta 314* (2001), 1–26.

[114] Machakova, I., Zazimalova, E., and George, E. F. *Plant Growth Regulators I: Introduction; Auxins, and their Analogues and Inhibitors.* Springer.

[115] Magnusson, C., Baron, J. A., Correia, N., Bergström, R., Adami, H.-O., and Persson, I. Breast-cancer risk following long-term oestrogen- and oestrogen-progestin-replacement therapy. *International Journal of Cancer 81* (1999), 339–344.

[116] Mannerås, L., Jonsdottir, I. H., Holmäng, A., Lönn, M., and Stener-Victorin, E. Low-frequency electro-acupuncture and physical exercise improve metabolic disturbances and modulate gene expression in adipose tissue in rats with dihydrotestosterone-induced polycystic ovary syndrome. *Endocrinology 149* (2008), 3559–3568.

[117] Mano, Y., and Nemoto, K. The pathway of auxin biosynthesis in plants. *Journal of Experimental Botany 63* (2012), 2853–2872.

[118] Manole, D., Schildknecht, B., Gosnell, B., Adams, E., and Derwahl, M. Estrogen promotes growth of human tyroid tumor cells by different molecular mechanisms. *The Journal of Clinical Endocrinology & Metabolism 86* (2001), 1072–1077.

[119] Mao, Q.-Q., Huang, Z., Zhong, X.-M., Xian, Y.-F., and Ip, S.-P. Piperine reverses the effects of corticosterone on behavior and hippocampal BDNF expression in mice. *Neurochemistry International 74* (2014), 36–41.

[120] Martinovic-Weigelt, D., Minarik, T. A., Curran, E. M., Marchuk, J. S., Pazderka, M. J., Smith, E. A., Goldenstein, R. L., Miresse, C. L., Matlon, T. J., Schultz, M. M., and Schoenfuss, H. L. Environmental estrogens in an urban aquatic ecosystem: I, spatial and temporal occurrence of estrogenic activity in effluent-dominated systems. *Environmental International 61* (2013), 138–149.

[121] Maser, E., and Xiong, G. The *Comamonas testosteroni* steroid biosensor system (COSS)-reflection on other methods. *Journal of Steroid Biochemistry & Molecular Biology 121* (2010), 633–640.

[122] Mavrodi, D. V., Bonsall, R. F., Delaney, S. M., Soule, M. J., Phillips, G., and Thomashow, L. S. Functional analysis of genes for biosynthesis of pyocyanin and phenazine-1-carboxamide from *Pseudomonas aeruginosa* PAO1. *Journal of Bacteriology 183* (2001), 6454–6465.

[123] Mavrodi, D. V., Peever, T. L., Mavrodi, O. V., Parejko, J. A., Raaijmakers, J. M., Lemanceau, P., Mazurier, S., Heide, L., Blankenfeldt, W., Weller, D. M., and Thomashow, L. S. Diversity and evolution of the phenazine biosynthesis pathway. *Applied and Environmental Microbiology 76* (2010), 866–879.

[124] Mazid, M., Ali, B., Hayat, S., and Ahmad, A. The effect of 4-chloroindole-3-acetic acid on some growth parameters in mung bean under cadmium stress. *Turkish Journal of Biology 34* (2010), 9–13.

[125] Möbus, E., and Maser, E. Molecular cloning, overexpression and characterization of steroid-inducible 3α-hydroxysteroid dehydrogenase/-carbonyl reductase from *Comamonas testosteroni*. *The Journal of Biological Chemistry 273* (1998), 30888–30896.

[126] Metcalfe, C. D., Metcalfe, T. L., Kiparissis, Y., Koenig, B. G., Khan, C., Hughes, R. J., Croley, T. R., March, R. E., and Potter, T. Estrogenic potency of chemicals detected in sewage treatment plant effluents as determined by *in vivo* assays with japanese medaka (*Oryzias latipes*). *Environmental Toxicology and Chemistry 20* (2001), 297–308.

[127] Michalowicz, J. Bisphenol A - sources, toxicity and biotransformation. *Environmental Toxicology and Pharmacology 37* (2014), 738–758.

[128] Miller, J. *Experiments in Molecular Genetics*.

[129] Mills, L. J., and Chichester, C. Review of evidence: Are endocrine-disrupting chemicals in the aquatic environment impacting fish populations. *Science of the Total Environment 343* (2005), 1–34.

[130] Mohammed, S. F., Ohtani, T., Korinek, J., Lam, C. S., Larsen, K., Simari, R. D., Valencik, M. L., Burnett, J. C., and Redfield, M. M. Mineralocorticoid accelerates transition to heart failure with preserved ejection fraction via "nongenomic effects". *Circulation 122* (2010), 370–377.

[131] Murray, F., Smith, D. W., and Hutson, P. H. Chronic low dose corticosterone exposure decreased hippocampal cell proliferation, volume and induced anxiety and depression like behaviours in mice. *European Journal of Pharmacology 583* (2008), 115–127.

[132] nero Fernandez, S. P., Chimerel, C., Keyser, U. F., and Summers, D. K. Indole transport across *Escherichia coli* membranes. *American Society for Microbiology 193* (2011), 1793–1798.

[133] Newman, J. D., and Setford, S. J. Enzymatic biosensors. *Molecular Biotechnology 32* (2006), 249–268.

[134] Nimrod, A. C., and Benson, W. H. Reproduction and development of japanese medaka following early life stage exposure to xenoestrogens. *Aquatic Toxicology 44* (1998), 141–156.

[135] Nivens, D. E., McKnight, T. E., Moser, S. A., Osbourn, S. J., Simpson, M. L., and Sayler, G. S. Bioluminescent bioreporter integrated circuits: potentially small, rugged and inexpensive whole-cell biosensors for remote environmental monitoring. *Journal of Applied Microbiology 96* (2004), 33–46.

[136] Norris, D. O. *Vertabrate Endocrinology*, fourth ed. Academic Press, 2007.

[137] Novak, O., Pencik, A., and Ljung, K. *Identification and Profiling of Auxin and Auxin Metabolites*. Springer.

[138] Ojeda, I., López-Montero, J., Moreno-Guzmán, M., Janegitz, B., González-Cortés, A., nez Sedeño, P. Y., and Pingarrón, J. Electrochemical immunosensor for rapid and sensitive determination of estradiol. *Analytica Chimica Acta 743* (2012), 117–124.

[139] Okuyama-Tamura, M., Mikuni, M., and Kojima, I. Modulation of the human glucocorticoid receptor function by antidepressive compounds. *Neuroscience Letters 342* (2003), 206–210.

[140] Oren, I., Fleishman, S. J., Kessel, A., and Ben-Tal, N. Free diffusion of steroid hormones across biomembranes: A simplex search with implicit solvent model calculations. *Biophysical Journal 87* (2004), 768–779.

[141] Örn, S., Holbech, H., Madsen, T. H., Norrgren, L., and Petersen, G. Gonad development and vitellogenin production in zebrafish (*Danio rerio*) exposed to ethinylestradiol and methyltestosterone. *Aquatic Toxicology 65* (2003), 397–411.

[142] Ortolá, A., Monerri, C., and Guardiola, J. The use of naphthalene acetic acid as a fruit growth enhancer in satsuma mandarin: a comparison with the fruit thinning effect. *Scientia Horticulturae 47* (1991), 15–25.

[143] Palmer, B. D., and Palmer, S. K. Vitellogenin induction by xenobiotic estrogens in the red-eared turtle and african clawed frog. *Environmental Health Perspectives 103* (1995), 19–25.

[144] Panter, G. H., Thompson, R. S., and Sumpter, J. P. Adverse reproductive effects in male fathead minnows (*Pimephales promelas*) exposed to environmentally relevant concentrations of the natural oestrogens, oestradiol and oestrone. *Aquatic Toxicology 42* (1998), 243–253.

[145] Parrella, A., Lavorgna, M., Criscuolo, E., Russo, C., and Isidori, M. Estrogenic activity and cytotoxicity of six anticancer drugs detected in water systems. *Science of the Total Environment 485-486* (2014), 216–222.

[146] Pastor, N., Rosas, S., Luna, V., and Rovera, M. Inoculation with *Pseudomonas putida* PC12, a phosphate solubilizing rhizobacterium, stimulates the growth of tomato plants. *Symbiosis 62* (2014), 157–167.

[147] Patt, H., Bandgar, T., Lila, A., and Shah, N. Management issues with exogenous steroid therapy. *Indian Journal of Endocrinology and Metabolism 17* (2013), S612–617.

[148] Pawlowski, S., van Aerle, R., Tyler, C. R., and Braunbeck, T. Effects of 17α-ethinylestradiol in fathead minnow (*Pimephales promelas*) gonadal recrudescence assay. *Ecotoxicology and Environmental Safety 57* (2004), 330–345.

[149] Peckett, A. J., Wright, D. C., and Riddell, M. C. The effects of glucocorticoids on adipose tissue lipid metabolism. *Metabolism Clinical and Experimental 60* (2011), 1500–1510.

[150] Pereira, R. O., Postigo, C., de Alda, M. L., Daniel, L. A., and Barceló, D. Removal of estrogens through water disinfection process and formation of by-products. *Chemosphere 82* (2011), 789–799.

[151] Pierson, L. S., and Pierson, E. A. Phenazine antibiotic production in *Pseudomonas aureofaciens*: role in rhizosphere ecology and pathogen suppression. *FEMS Microbiology Letters 136* (1996), 101–108.

[152] Pope, H. G., Kouri, E. M., and Hudson, J. I. Effects on supraphysiological doses of testosterone on mood and aggression in normal men. *Archives of General Psychiatry 57* (2000), 133–140.

[153] Priego-Capote, F., Orozco-Solano, M. I., and Calderón-Santiago, M. Quantitative determination and confirmatory analysis of

n-acetylneuraminic and n-glycolylneuraminic acids in serum and urine by solid-phase extraction on-line coupled to liquid chromatography-tandem mass spectrometry. *Journal of Chromatography A 1346* (2014), 88–96.

[154] Prieto, M. A., Díaz, E., and García, J. L. Molecular characterization of the 4-hydroxyphenylacetate catabolic pathway of *Escherichia coli* W: Engineering a mobile aromatic degradative cluster. *Journal of Bacteriology 178* (1996), 111–120.

[155] Prieto, M. A., and Garcia, J. L. Identification of a novel positive regulator of the 4-hydroxyphenylacetate catabolic pathway of *Escherichia coli*. *Biochemical and Biophysical Research Communications 232* (1997), 759–765.

[156] Prieto, M. A., and García, J. L. Identification of the 4-hydroxyphenylacetate transport gene of *Escherichia coli* W: construction of a highly sensitive cellular biosensor. *Federation of European Biochemical Societies Letters 414* (1997), 293–297.

[157] Purdom, C., Hardiman, P., Bye, V., Eno, N., Tyler, C., and Sumpter, J. Estrogenic effects of effluents from sewage treatment works. *Chemistry and Ecology 8* (1994), 275–285.

[158] Rada, B., and Leto, T. L. Pyocyanin effects on respiratory epithelium: relevance in *Pseudomonas aeruginosa* airway infections. *Trends in Microbiology 21* (2013), 73–81.

[159] Rebarber, A., Istwan, N. B., Russo-Stieglitz, K., Cleary-Goldman, J., Rhea, D. J., Stanziano, G., and Saltzman, D. H. Increased incidence of gestational diabetes in women receiving prophylactic 17α-hydroxyprogesterone caproate for prevention of recurrent preterm delivery. *Diabetes Care 30* (2007), 2277–2280.

[160] Rebuffé-Scrive, M., Walsh, U., McEwen, B., and Rodin, J. Effect of chronic stress and exogenous glucocorticoids on regional fat distribution and metabolism. *Physiology and Behavior 52* (1992), 583–590.

[161] Rivera, R., Yacobson, I., and Grimes, D. The mechanism of action of hormonal contraceptives and intrauterine contraceptives devices. *American Journal of Obstetrics and Gynecology 181* (1999), 1263–1269.

[162] Rocha, R., and Jr., C. T. S. Pathophysiological effects of aldosterone in cardiovascular tissues. *TRENDS in Endocrinology & Metabolism 12* (2001), 308–314.

[163] Ross, R. K., Paganini-Hill, A., Wan, P. C., and Pike, M. C. Effect of hormone replacement therapy on breast cancer risk: Estrogen versus estrogen plus progestin. *Journal of the National Cancer Institute 92* (2000), 328–332.

[164] Routledge, E. J., and Sumpter, J. P. Estrogenic activity of surfactants and some of their degradation products assessed using a recombinant yeast screen. *Environmental Toxicology and Chemistry 15* (1996), 241–248.

[165] Royer, P. A., and Jones, K. P. Progestins for contraception: Modern delivery systems and novel formulations. *Clinical Obstetrics and Gynecology 57* (2014), 644–658.

[166] Salamon, Z., Macleod, H. A., and Tollin, G. Surface plasmon resonance spectroscopy as a tool for investigating the biochemical and biophysical properties of membrane protein systems. II: Applications to biological systems. *Biochimica et Biophysica Acta 1331* (1997), 131–152.

[167] Sambrook, J., and Russell, D. *Molecular cloning: a laboratory manual*, third ed. Cold Spring Harbor Laboratory Press, 2001.

[168] Santner, A., Calderon-Villalobos, L. I. A., and Estelle, M. Plant hormones are versatile chemical regulators of plant growth. *Nature Chemical Biology 5* (2009), 301–307.

[169] Schultz, M. M., Minarik, T. A., Martinovic-Weigelt, D., Curran, E. M., Bartell, S. E., and Schoenfuss, H. L. Environmental estrogens in an urban aquatic ecosystem: II, biological effects. *Environmental International 61* (2013), 138–149.

[170] Scott, J. C., Greenhut, I. V., and Leveau, J. H. J. Functional characterization of the bacterial *iac* genes for degradation of the plant hormone indole-3-acetic acid. *Journal of Chemical Ecology 39* (2013), 942–951.

[171] Seki, M., Yokota, H., Maeda, M., and Kobayashi, K. Fish full life-cycle testing for 17β-estradiol on medaka (*Oryzias Latipes*). *Environmental Toxicology and Chemistry 24* (2005), 1259–1266.

[172] Serrano, L. Synthetic biology: promises and challenges. *Molecular Systems Biology 3* (2007), 1–5.

[173] Sharifi, G., and Ebrahimzadeh, H. Interaction of IBA and NAA with enzymes in root induction of *Crocus sativus* (l). *African Journal of Biotechnology 9* (2010), 217–225.

[174] Shingler, V., and Moore, T. Sensing of aromatic compounds by the DmpR transcriptional activator of phenol-catabolizing *Pseudomonas* sp. strain CF600. *Journal of Bacteriology 176* (1994), 1555–1560.

[175] Shore, L. S., and Shemesh, M. Naturally produced steroid hormones and their release into the environment. *Pure and Applied Chemistry 75*, 11-12 (2003), 1859–1871.

[176] Siegele, D. A., and Hu, J. C. Gene expression from plasmids containing the *araBAD* promoter at subsaturating inducer concentrations represents mixed populations. *Proceedings of the National Academy of Sciences 94* (1997), 8168–8172.

[177] Sirbasku, D. A. Estrogen induction of growth factors specific for hormone-responsive mammary, pituitary, and kidney tumor cells. *Proceedings of the National Academy of Sciences of the USA 75* (1978), 3786–3790.

[178] Smeets, J. M. W., Rankouhi, T. R., Nichols, K. M., Komen, H., Kaminski, N. E., Giesy, J. P., and van den Berg, M. *In Vitro* vitellogenin production by carp (*Cyprinius carpio*) hepatocytes as a screening method for determining (anti)estrogenic activity of xenobiotics. *Toxicology and Applied Pharmacology 157* (1999), 68–76.

[179] Smolke, C. D. Building outside of the box: iGEM and the BioBricks Foundation. *Nature Biotechnology 27* (2009), 1099–1102.

[180] Snow, A., Andow, D., Gepts, P., Hallerman, E., Power, A., Tiedje, J., and Wolfenbarger, L. Genetically engineered organisms and the environment: current status and recommendations. *Ecological Applications 15* (2005), 377–404.

[181] Soto, A. M., Justicia, H., Wray, J. W., and Sonnenschein, C. p-nonyl-phenol: An estrogenic xenobiotic released from modified polystyrene. *Environmental Health Perspectives 92* (1991), 167–173.

[182] Soto, A. M., Sonnenschein, C., Chung, K. L., Fernandez, M. F., Olea, N., and Serrano, F. O. The E-SCREEN assay as a tool to identify estrogens: An update on estrogenic environmental pollutants. *Environmental Health Perspectives 103* (1995), 113–122.

[183] Spaepen, S., Vanderleyden, J., and Remans, R. Indole-3-acetic acid in microbial and microorganism-plant signaling. *FEMS Microbiology Reviews 31* (2007), 425–448.

[184] Sticher, P., Jaspers, M. C., Stemmler, K., Harms, H., Zehnder, A. J., and van der Meer, J. R. Development and characterization of a whole-cell bioluminescent sensor for bioavailable middle-chain alkanes in contaminated groundwater samples. *Applied and Environmental Microbiology 63* (1997), 4053–4060.

[185] Sumpter, J. P., and Jobling, S. The occurence, causes, and consequences of estrogens in the aquatic environment. *Environmental Toxicology and Chemistry 32*, 2 (2013), 249–251.

[186] Sun, B., Chen, L., Xu, Y., Liu, M., Yin, H., and Ai, S. Ultrasensitive photoelectrochemical immunoassay of indole-3-acetic acid based on the MPA modified CdS/RGO nanocomposites decorated ITO electrode. *Biosensors and Bioelectronics 51* (2014), 164–169.

[187] Tamaoka, J., Ha, D.-M., and Komagata, K. Reclassification of *Pseudomonas acidovorans* den dooren de jong 1926 and *Pseudomonas testosteroni* marcus and talalay 1956 as *Comamonas acidovorans* comb. nov. and *Comamonas testosteroni* comb. nov., with an emended description of the genus *Comamonas*. *International Journal of Systematic Bacteriology 37* (1987), 52–59.

[188] Tang, B., Xi, C., Zou, Y., Wang, G., Li, X., Zhang, L., Chen, D., and Zhang, J. Simultaneous determination of 16 synthetic colorants in hotpot condiment by high performance liquid chromatography. *Journal of Chromatography B 960* (2014), 87–91.

[189] tanimoto, E. Regulation of root growth by plant hormones–roles for auxin and gibberellin. *Critical Reviews in Plant Sciences 24* (2005), 249–265.

[190] Tchaikovski, S. N., and Rosing, J. Mechanisms of estrogen-induced venous thromboembolism. *Thrombosis Research 126* (2010), 5–11.

[191] Tecon, R., and van der Meer, J. R. Bacterial biosensors for measuring availability of environmental pollutants. *Sensors 8* (2008), 4062–4080.

[192] Teede, H., Deeks, A., and Moran, L. Polycystic ovary syndrome: a complex condition with psychological, reproductive and metabolic manifestations that impacts on health across the lifespan. *Biomed Central Medicine 8* (2010), 41–50.

[193] Ternes, T., Stumpf, M., Mueller, J., Haberer, K., Wilken, R.-D., and Servos, M. Behavior and occurrence of estrogens in municipal sewage treatment plants — I. investigations in Germany, Canada and Brazil. *The Science of the Total Environment 225* (1999), 81–90.

[194] Tiedje, J. M., Colwell, R. K., Grossman, Y. L., Hodson, R. E., Lenski, R. E., Mack, R. N., and Regal, P. J. The planned introduction of genetically engineered organisms: ecological considerations and recommendations. *Ecology 70* (1989), 298–315.

[195] Toppari, J., Larsen, J. C., Christiansen, P., Giwercman, A., Grandjean, P., Guillette, L. J., Jégou, B., Jensen, T. K., Jouannet, P., Keiding, N., Leffers, H., McLachlan, J. A., Meyer, O., Müller, J., Meyts, E. R.-D., Scheike, T., Sharpe, R., Sumpter, J., and Skakkebæk, N. E. Male reproductive health and environmental xenoestrogens. *Environmental Health Perspectives 104* (1996), 741–803.

[196] Trögl, J., Ripp, S., Kuncová, G., Sayler, G. S., Churavá, A., Paříк, P., Demnerová, K., Hálová, J., and Kubicová, L. Selectivity of whole cell optical biosensor with immobilized bioreporter *Pseudomonas fluorescens* HK44. *Sensors and Actuators 107* (2005), 98–103.

[197] Turner, J. M., and Messenger, A. J. Occurrence, biochemistry and physiology of phenazin pigment production. *Advances in Microbial Physiology 27* (1986), 211–286.

[198] Ullah, Z., Abbas, S. J., Naeem, N., Luftfullah, G., Malik, T., and Khan, M. A. U. K. I. Effect of indolebutyric acid (IBA) and naphthaleneacetic acid (NAA) plant growth regulaters on mari gold (*Tagetes erecta* l.). *African Journal of Agricultural Research 8* (2013), 4015–4019.

[199] United Nations. *World Water Development Report 2003*. `http://www.unwater.org/publications/world-water-development-report/en/`.

[200] United Nations. *World Water Development Report 2014*. `http://unesdoc.unesco.org/images/0022/002257/225741E.pdf`.

[201] United Nations Department of Economic and Social Affairs. *World urbanization prospects*. `http://esa.un.org/unpd/wup/CD-ROM/Urban-Rural-Population.htm`.

[202] United Nations, Department of Economic and Social Affairs, Population Division (2012). *World Contraceptive Use 2012 (POP/DB/CP/Rev2012)*.

[203] Valdivia, R. H., and Falkow, S. Bacterial genetics by flow cytometry: rapid isolation of *Salmonella typhimurium* acid-inducible promoters by differential fluorescence induction. *Molecular Microbiology 22* (1996), 367–378.

[204] Van den Belt, K., Berckmans, P., Vangenechten, C., Verheyen, R., and Witters, H. Comparative study on the in vitro/in vivo estrogenic potencies of 17β-estradiol, estrone, 17α-ethinylestradiol and nonylphenol. *Aquatic Toxicology 66* (2004), 183–195.

[205] van der Meer, J. R., and Belkin, S. Where microbiology meets microengineering: design and applications of reporter bacteria. *Nature review 8* (2010), 1788–1799.

[206] van der Meer, J. R., Tropel, D., and Jaspers, M. Illuminating the detection chain of bacterial bioreporters. *Environmental Microbiology 6* (2004), 1005–1020.

[207] Védrine, C., leclerc, J.-C., Durrieu, C., and Tran-Minh, C. Optical whole-cell biosensor using *Chlorella vulgaris* designed for monitoring herbicides. *Biosensors and Bioelectronics 18* (2003), 457–463.

[208] Venken, L. Pyocyanin: electrical connection between bacteria and microchips. Master's thesis, Faculty of Bioscience Engineering, 2014.

[209] Verliefde, A., Cornelissen, E., Amy, G., Van der Bruggen, B., and van Dijk, H. Priority organic micropollutants in water sources in Flanders and the Netherlands and assessment of removal possibilities with nanofiltration. *Environmental Pollution 146* (2007), 281–289.

[210] Vélez, M. P. Functional analysis of probiotic characteristics of *Lactobacillus rhamnosus* GG. Master's thesis, Faculty of Bioscience Engineering, 2007.

[211] Vulliet, E., Wiest, L., Baudot, R., and Grenier-Loustalot, M.-F. Multi-residue analysis of steroids at sub-ng/l levels in surface and ground-waters using liquid chromatography coupled to tandem mass spectrometry. *Journal of Chromatography A 1210* (2008), 84–91.

[212] Warner, D. A., Addis, E., guo Du, W., Wibbels, T., and Janzen, F. J. Exogenous application of estradiol to eggs unexpectedly induces male development in two turtle species with temperature-dependent sex determination. *General and Comparative Endocrinology 206* (2014), 16–23.

[213] Warris, L., van den Heuvel-Eibrink, M., den Hoed, M., Aarsen, F., Pieters, R., and van den Akker, E. Does dexamethasone induce more neuropsychological side effects than prednisone in pediatric acute lymphoblastic leukemia? a sytematic review. *Pediatric Blood & Cancer 61* (2014), 1313–1318.

[214] Watson, C. S., Bulayeva, N. N., Wozniak, A. L., and Alyea, R. A. Xenoestrogens are potent activators of nongenomic estrogenic responses. *Steroids 72* (2007), 124–134.

[215] Webster, T. A., Sismaet, H. J., Conte, J. L., ping J. Chan, I., and Goluch, E. D. Electrochemical detection of *Pseudomonas aeruginosa* in human fluid samples via pyocyanin. *Biosensors and Bioelectronics 60* (2014), 265–270.

[216] Weinstein, R. S. Glucocorticoid-induced osteoporosis and osteonecrosis. *Endocrinology Metabolism Clinics of North America 41* (2012), 595–611.

[217] Weyers, J. D., and Paterson, N. W. Plant hormones and the control of phyiological processes. *New Phytologist 151* (2001), 375–407.

[218] Willardson, B. M., Wilkins, J. F., Rand, T. A., Schupp, J. M., Hill, K. K., Keim, P., and Jackson, P. J. Development and testing of a bacterial biosensor for toluene-based environmental contaminants. *Applied and Environmental Microbiology 64* (1998), 1006–1012.

[219] Xiong, G., Luo, Y., Jin, S., and Maser, E. *Cis*- and *trans*-regulatory elements of 3α-hydroxysteroid dehydrogenas/carbonyl reductase as biosensor system for steroid determination in the environment. *Chemico-Biological Interactions 178* (2009), 215–220.

[220] Xiong, G., Martin, H.-J., Blum, A., Schäfers, C., and Maser, E. A model on the regulation of 3α-hydroxysteroid dehydrogenase/carbonyl reductase expression in *Comamonas testosteroni*. *Chemico-Biological Interactions 130-132* (2001), 723–736.

[221] Xiong, G., Martin, H. J., and Maser, E. Identification and characterization of a novel translational repressor of the steroid-inducible 3α-hydroxysteroid dehydrogenas/carbonyl reductase gene in *Comamonas testosteroni*. *Journal of Biological Chemistry 278* (2003), 47400–47407.

[222] Xita, N., and Tsatsoulis, A. Review: Fetal programming of polycystic ovary syndrome by androgen excess: Evidence from experimental, clinical, and genetic association studies. *The Journal of Clinical Endocrinology & Metabolism 91* (2006), 1660–1666.

[223] Xue, B., Zhang, Z., Roncari, C. F., Guo, F., and Johnson, A. K. Aldosterone acting through the cns sensitizes angiotensin II - induced hypertension. *Hypertension 60* (2012), 1023–1030.

[224] Yager, J. D., and Davidson, N. E. Estrogen carcinogenesis in breast cancer. *The New England Journal of Medicine 354* (2006), 270–282.

[225] Yan, Y.-H., Li, J.-L., Zhang, X.-Q., Yang, W.-Y., Wan, Y., Ma, Y.-M., Zhu, Y.-Q., Peng, Y., and Huang, L.-K. Effects of naphthalene acetic acid on adventitious root development and associated physiological changes in stem cutting of *Hemarthria compressa*. *Plos One 9* (2014), 1–6.

[226] Yanisch-Perron, C., Vieira, J., and Messing, J. Improved M13 phage cloning vectors and host strains: nucleotide sequencing of the M13mp18 and pUC19 vectors. *Gene 33* (1985), 103–119.

[227] Yin, X.-B., Guo, J.-M., and Wei, W. Dual-cloud point extraction and tertiary amine labeling for selective and sensitive capillary electrophoresis-electrochemiluminescent detection of auxins. *Journal of Chromatography 1217* (2010), 1399–1406.

[228] Ying, G.-G., Kookana, R. S., Kumar, A., and Mortimer, M. Occurrence and implications of estrogens and xenoestrogens in sewage effluents and receiving waters from South East Queensland. *Science of the Total Environment 407* (2009), 5147–5155.

[229] Ying, G.-G., Kookana, R. S., and Ru, Y.-J. Occurrence and fate of hormone steroids in the environment. *Environment International 28* (2002), 545–551.

[230] Zhang, Q., Wang, Y., Mateescu, A., Sergelen, K., Kibrom, A., Jonas, U., Wei, T., and Dostalek, J. Biosensor based on hydrogel waveguide spectroscopy for the detection of 17β-estradiol. *Talanta 104* (2013), 149–154.

[231] Zhao, Y. Auxin biosynthesis and its role in plant development. *Annual Review of Plant Biology 61* (2010), 49–64.

[232] Zhao, Y., Boyd, J. M., Sawyer, M. B., and Li, X.-F. Liquid chromatography tandem mass spectrometry determination of free and conjugated estrogens in breast cancer patients before and after exemestane treatment. *Analytica Chimica Acta 806* (2014), 172–179.

[233] Zhou, Y., Xu, Z., Meng, X., and Yin, H. Electrochemical immunoassay platform for high sensitivity detection of indole-3-acetic acid. *Electrochimica Acta 96* (2013), 66–73.

[234] Ziauddin, A., Marsolais, A., Simion, E., and Kasha, K. Improved plant regeneration from wheat anther and barley microspore culture using phenylacetic acid (PAA). *Plant Cell Reports 11* (1992), 489–498.

[235] Zoeller, R. T., Brown, T., Doan, L., Gore, A., Skakkebæk, N., Soto, A., Woodruff, T., and Saal, F. V. Endocrine-disrupting chemicals and public health protection: A statement of principles from the endocrine society. *Endocrinology 153* (2012), 4097–4110.

List of Publications

Articles in internationally reviewed academic journals

DIERCKX, S., VAN PUYVELDE, S., VENKEN, L., EBERLE, W., AND VANDERLEYDEN, J. Design and construction of a whole cell bacterial 4-hydroxyphenylacetic acid and 2-phenylacetic acid bioassay. *Frontiers in Bioengineering and Biotechnology 3* (2015)

BERDAKIN, I., JEYARAM, Y., MOSHCHALKOV, V., VENKEN, L., DIERCKX, S., VANDERLEYDEN, J., SILHANEK, A., CONDAT, C., AND MARCONI, V. Influence of swimming strategy on microorganism separation by asymmetric obstacles. *Phys. Rev. E. Stat. Nonlin. Soft Matter Phys. 87* (2013)

Meeting abstracts, presented at international scientific conferences and symposia

DIERCKX, S., VANDERLEYDEN, J., EBERLE, W. Synthetic Biology: design and construction of a steroid biosensor. Young Microbiologists Symposium. Cork, Ireland, 21-22 June 2012.
DIERCKX, S., VANDERLEYDEN, J., EBERLE, W. Synthetic Biology: design and construction of a steroid biosensor. Biosensors. Cancun, Mexico, 15-18 May 2012
DIERCKX, S., VANDERLEYDEN, J., EBERLE, W. Synthetic Biology: design and construction of a steroid biosensor. SB6.0: The Sixth International Meeting on Synthetic Biology. London, United Kingdom, 9-11 July 2013

FACULTY OF BIOSCIENCE ENGINEERING
DEPARTMENT OF MICROBIAL AND MOLECULAR SYSTEMS
CENTRE OF MICROBIAL AND PLANT GENETICS
Kasteelpark Arenberg 20
B-3001 Heverlee
seppe.dierckx@biw.kuleuven.be